(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
29 March 2001 (29.03.2001)

PCT

(10) International Publication Number
WO 01/21204 A1

(51) International Patent Classification[7]: A61K 39/12, 39/42, C07H 21/04, C07K 16/08, C12N 15/11, C12Q 1/70, G01N 33/53

(21) International Application Number: PCT/US00/25871

(22) International Filing Date:
20 September 2000 (20.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/154,799 20 September 1999 (20.09.1999) US

(71) Applicants: **THE JOHN P. ROBARTS RESEARCH INSTITUTE** [CA/CA]; 100 Perth Drive, London, Ontario M6A 5K8 (CA). **WASHINGTON UNIVERSITY** [US/US]; St. Louis, MO 63130 (US).

(72) Inventors: **MCFADDEN, Grant**; 1435 Corley Drive S., London, Ontario N6G 2K5 (CA). **SPECK, Samuel**; 1921 Windy Hill Road, St. Louis, MO 63122 (US). **VIRGIN, Herbert, IV**; 5082 Washington Place, St. Louis, MO 63108 (US). **LUCAS, Alexandra**; P.O. Box 5015, London, Ontario N6A 5K8 (CA).

(74) Agent: **BIEKER-BRADY, Kristina**; Clark & Elbing LLP, 176 Federal Street, Boston, MA 02110-2214 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/21204 A1

(54) Title: THERAPEUTIC USES OF M3 POLYPEPTIDE

(57) Abstract: The present invention features methods of using M3 polypeptide formulated for the treatment of a variety of inflammatory and autoimmune disorders.

THERAPEUTIC USES OF M3 POLYPEPTIDE

FIELD OF THE INVENTION

This invention generally relates to a viral protein for use in the treatment of human diseases. The viral protein, known as M3, was initially isolated from the gammaherpesvirus-68 ("γHV68"). This invention is based, in part, on the discovery that M3 protein binds various biologically-relevant compounds within the human organism and mediates an anti-inflammatory and/or immunomodulatory role when administered *in vivo*.

BACKGROUND OF THE INVENTION

Viruses propagate by living within cells of higher-order vertebrates. Accordingly, they have evolved specifically to avoid the host immune system. In fact, virus survival is dependent upon strategies to evade, suppress, counteract, or otherwise circumvent the myriad of host responses to a foreign invader. The selective pressure conferred by the effector elements of the immune system of the host can clearly be a powerful element of evolutionary pressure; in fact all eukaryotic viruses existing today contain imprints or remnants of their battles with the immune system as evidenced by the presence of encoded proteins that suppress the immune system or as evidenced by survival strategies that allow the virus to evade immune system detection.

The specific strategy or strategies used by a virus varies dramatically according to its genome capacity. Viruses with small genomes ensure their survival by exploiting weaknesses or gaps in the host immune repertoire to evade detection. Alternatively, the smaller genome replicates very rapidly, effectively outpacing the immune response. The larger DNA viruses (*i.e.* the adenoviruses, herpesviruses, iridoviruses and poxviruses) specifically encode proteins that function to protect the virus from immune recognition and/or clearance by the infected host. Such "subversive" viral proteins are useful therapeutics for the treatment of inflammatory and auto-immune disorders.

One such protein within the γHV68 genome, M3 protein, is encoded by the third open reading frame of γHV68 and constitutes 406 amino acid residues (Virgin H.W. et

al. Complete sequence and genomic analysis of murine gammaherpesvirus 68. J Virol. 1997. 71(8):5894-904). The M3 gene is located between nucleotides 6060 and 7277 within the γHV68 genome.

Host and viral immunomodulatory proteins include chemotactic cytokines, called "chemokines." Chemokines are small molecular weight immune ligands that are chemoattractants for leukocytes, such as neutrophils, basophils, monocytes, and T cells. There are two major classes of chemokines, both of which contain four conserved cysteine residues that form disulfide bonds in the tertiary structure of the proteins. The class is designated C-X-C (where X is any amino acid), and includes IL-8, CTAP-III, gro/MGSA and ENA-78; and the class, designated C-C, includes MCP-1, MIP-1, and Regulated on Activation, Normal T Expressed and Secreted protein (RANTES). The designations of the classes are according to whether an intervening residue spaces the first two cysteines in the motif. In general, most C-X-C chemokines are chemoattractants for neutrophils, but not monocytes, whereas C-C chemokines appear to attract monocytes and lymphocytes, but not neutrophils. Recently, a third group of chemokines, the "C" group, was designated by the discovery of a new protein called lymphotactin. A fourth group of chemokines, the CX_3C family, includes the chemokine fractalkine. The chemokine families is believed to be critically important in the infiltration of lymphocytes and monocytes into sites of inflammation. Chemokines are known to play a significant role in a variety of different diseases, including but not limited to, allergenic inflammation, diabetes mellitus, rheumatoid arthritis, multiple sclerosis, transplant rejection, and glomerulonephritis.

Both the host and virus may also encode a class of proteins that regulate apoptosis (or cell death). The relationship between viruses and apoptosis is complex, in part because all viruses are faced with the need to first productively replicate within a receptive intracellular environment, then to spread to other cells or tissues, and finally to seek out new hosts in order to continue the chain of propagation. Thus, there are times when the premature induction of apoptosis can be detrimental to the virus, for example, by disrupting the viral replicative life cycle, while at other times apoptosis can become advantageous, for example, by facilitating virus dissemination and secondary infection of phagocytes or other immune cells. As viruses have acquired mechanisms to evade the host's immune system, viruses have collectively evolved strategies to either inhibit or stimulate apoptosis, depending on the particular virus/host interaction.

The gammaherpesvirus family is characterized biologically by the ability of its members to infect and establish latency within lymphocytes. Furthermore, members of the gammaherpesvirus family are also associated with tumors in immunosuppressed host organisms. Among the various members of the gammaherpesvirus family, γHV68 is a natural pathogen of out-bred and in-bred mice. Upon establishment of latency, γHV68 causes several different chronic diseases in immunocompromised mice, including a severe vasculitis and lymphoproliferative disease. The γHV68 virus is also known to infect several organs within murine hosts and can establish a latent, persistent infection within the spleen.

Little is known regarding the various mechanisms utilized by γHV68 in establishing latency and evading host immune responses during infection. The γHV68 genome has been fully sequenced and published within the literature (Virgin H.W. et al. Complete sequence and genomic analysis of murine M3. J Virol. 1997. 71(8):5894-904). The complete viral genome of γHV68 encodes at least 80 genes of various different sizes. Since the vast majority of these proteins demonstrate poor homology to all known genes and indicate little functional similarity to other viral protein sequences, the biological function of these 80 genes is yet to be elucidated.

There exists a need for new drugs that are useful for the treatment of human inflammatory and autoimmune disorders. Identification of novel immunomodulatory agents may reveal new pharmaceuticals that can be used to suppress inflammation and dysregulation of the immune system. In addition, novel agents provide new probes and targets that can be used to identify novel elements of the cellular immune repertoire and new classes of drugs. Such tools may promote understanding of the molecular mechanisms by which viral immunomodulators function and may reveal targets for pharmacological intervention that could be of substantial benefit in treating these diseases.

SUMMARY OF THE INVENTION

In general the present invention features an immunomodulatory protein that may be used in the treatment of a variety of immunomodulatory disorders. The present invention describes functional properties of the M3 protein encoded and expressed by γHV68. In particular, the present invention features a M3 polypeptide capable of

binding various chemokines that function in chemotactic recruitment of leukocytes. The present invention also describes the binding of M3 protein to chemokines at very high affinity and discloses that M3 protein is a ligand of said chemokines. The present invention also describes the administration of M3 protein to a mammalian organism in order to bind chemokine molecules *in vivo* and thereby inhibit their function in recruitment and chemotaxis of leukocytes.

In one aspect, the invention features a method of immunomodulation in a mammal, by administering to the mammal a therapeutically effective amount of a M3 polypeptide, where the polypeptide has an immunomodulatory effect in the mammal. In another aspect, the invention features a method of immunomodulation wherein the method involves providing the M3 gene under the control of a promoter providing controllable expression of the M3 gene in a cell wherein the M3 gene is expressed in a construct capable of delivering a M3 protein in an amount effective to decrease inflammation or inhibit autoimmune reaction. The polypeptide may also be provided directly, for example, in cell culture, for therapeutic uses. In preferred embodiments, M3 polypeptide is delivered by expression of the M3 gene using a tissue-specific or cell type-specific promoter, or by a promoter that is activated by the introduction of an external signal or agent, such as a chemical signal or agent. In preferred embodiments, the method is used for improving prognosis in patients with tumors. The method includes providing a M3 protein that binds chemokines, which induce or prevent apoptosis in the region of the tumor either by providing an effective amount of the polypeptide or by providing an effective amount of a transgene expressing the polypeptide. In one such embodiment the tumor is a solid tumor, *e.g.* lymphoma (*e.g.*, Hodgkin's), plasmacytoma, carcinoma (*e.g.*, gastric, colonic, and lung carcinomas), melanoma, and sarcoma.

In another aspect, the invention features a method of immunomodulation in a mammal, by administering to the mammal a therapeutically effective amount of an M3 encoding nucleic acid compound that encodes M3 polypeptide sufficient to modulate chemokine activity, where the M3 has an immunomodulatory effect in the mammal. The introduction of nucleic acid molecules into cells and tissues may be utilized to amplify the transcription and translation of M3 polypeptide-encoding nucleic acids or related truncations, analogs or homologs or related nucleic acid sequences, such as SEQ ID NO:1 or SEQ ID NO:2. Alternatively, M3 polypeptide expression may be

downregulated by the introduction of complementary nucleic acid sequences that block transcription and translation of M3 protein-encoding nucleic acids or related truncations, analogs or homologs or related nucleic acid sequences such as SEQ ID NO:1 or SEQ ID NO:2.

In related aspects, the present invention provides a method for treating AIDS, cirrhosis of the liver, neurodegeneration, myelodysplastic syndrome or ischemic injury by administration of a M3 polypeptide.

In other preferred embodiments of some of the above aspects, the immunomodulation includes immunosuppression, immunostimulation, cell proliferation, apoptosis, decreasing T cell stimulation, or decreasing inflammation in a mammal (*e.g.*, a human). The mammal may be diagnosed with a tumor (*e.g.*, a carcinoma, a plasmacytoma, a lymphoma, or a sarcoma).

In other preferred embodiments of some of the above aspects, the mammal has a condition selected from the group consisting of acute inflammation, rheumatoid arthritis, transplant rejection, restenosis, asthma, allergies, inflammatory bowel disease, uveitis, psoriasis, atopic dermatitis, bronchial asthma, pollinosis, systemic lupus erythematosus, nephrotic syndrome lupus, multiple sclerosis, myasthenia gravis, type I and type II diabetes mellitus, glomerulonephritis, Hashimoto's thyroiditis, autoimmune hemolytic anemia, autoimmune thrombocytopenic purpura, microbial infection, malignancy and metastasis, autoimmune disease, cirrhosis, endotoxemia, atherosclerosis, reperfusion injury and inflammatory responses, AIDS, cirrhosis of the liver, neurodegeneration, myelodysplastic syndrome, and ischemic injury. In a particularly preferred embodiment, the mammal has a neurological condition, such as a central nervous system infection or disease. It is particularly preferred that the mammal be diagnosed with an inflammatory or autoimmune disease (as those listed herein above). Alternatively, in other preferred embodiments, the mammal has a tumor (*e.g.*, a carcinoma, a plasmacytoma, a lymphoma or a sarcoma).

In another aspect, the invention features a method of reducing inflammation. For example, damage occurring during asthmatic reactions, inflammatory bowel diseases (*i.e.*, Crohn's Disease and ulcerative colitis), eosinophilic colitis, or allergic rhinitis. The method includes inhibiting inflammation normally caused by an immunostimulatory chemokine. In some embodiments, the antagonist is a M3 polypeptide having a deletion of amino acids or having an addition of amino acids on the carboxy or alternatively the

amino terminus. Where amino acids are added they may be random or they may be selected to have particular biological properties such as stability or hydrophilicity.

In related aspects, the methods of the present invention include administering a M3 polypeptide by providing a cell that expresses a M3 nucleic acid (*i.e.*, a transgenic cell). In various embodiments, the nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:2. In a preferred embodiment, the cell is a transformed human cell. These sequences may also be expressed in non-human species including but not limited to bacteria, yeast, zebrafish, xenopus, drosophila, mice, rats, rabbits, sheep, pigs, and chickens. In another preferred embodiment, a cell that expresses a M3 nucleic acid provided to a patient needing treatment in the form of immunomodulation (*e.g.*, immunosuppression or immunostimulation). Alternatively, the patient is experiencing any of a wide variety of inflammatory or autoimmune disorders (*e.g.*, an allergic reaction, acute inflammation, rheumatoid arthritis transplant rejection, restenosis, asthma, uveitis or inflammatory bowel disease).

In another aspect, the invention features a method of inhibiting hyperplasia in a mammal. For example, in response to vascular injury, chemokines are upregulated during restenosis, which leads to recurrent atheroscerotic plaque growth. The method includes administering a therapeutically effective amount of a M3 polypeptide to a mammal having intimal hyperplasia such that the intimal hyperplasia is inhibited.

In another aspect, the invention features a M3 polypeptide which can bind chemokines in a pharmaceutically acceptable carrier. For example, the M3 polypeptide having SEQ ID NO:3 may bind one or more types of chemokines derived from or present within mammalian organisms. In a related aspect, the M3 polypeptide may be formulated for administration *in vivo* to bind one or more chemokine proteins within mammalian organisms. Furthermore, the M3 polypeptide may be administered or expressed within specific mammalian tissues to bind chemokines present within said tissue. It is envisioned herein that the binding between M3 polypeptide and a chemokine shall reduce, inhibit and/or other diminish the ability of said chemokine to conduct its normal function within mammalian organisms. The pharmaceutical compositions which include a therapeutically effective amount of either an M3 polypeptide or an M3 nucleic acid may have, in a preferred embodiment, M3 polypeptide having the amino acid sequence of SEQ ID NO:3. In another preferred embodiment, the M3 nucleic acid has the nucleotide sequence of either SEQ ID NO:1 or SEQ ID NO:2.

In another aspect, the invention features a diagnostic kit for detecting the presence of M3 polypeptide in a patient. In a preferred embodiment, the diagnostic kit includes an M3 primer. In another preferred embodiment, the diagnostic kit includes an antibody that specifically binds an M3 polypeptide.

Definitions

"Polypeptide" or "polypeptide fragment" means a chain of two or more amino acids, regardless of any post-translational modification (*e.g.*, glycosylation or phosphorylation), constituting all or part of a naturally or non-naturally occurring polypeptide. By "post-translational modification" is meant any change to a polypeptide or polypeptide fragment during or after synthesis. Post-translational modifications include, but are not limited to, glycosylation, myristylation, tyrosine phosphorylation, serine phosphorylation, threonine phosphorylation, ubiquitination and proteolytic degradation. Post-translational modifications can be produced naturally (such as during synthesis within a cell) or generated artificially (such as by recombinant or chemical means). A "protein" can be made up of one or more polypeptides.

"M3 immunomodulatory polypeptide" or "M3 polypeptide" includes the protein encoded by the third open reading frame of murine γHV68, as well as fragments and homologs thereof. The M3 polypeptide used in the present invention may be prepared by a variety of techniques known in the art. A preferred method of preparing M3 polypeptide is recombinant expression, for example by transforming cell lines with the nucleic acid sequence of SEQ ID NO:1 or SEQ ID NO:2. A preferred M3 polypeptide used in the invention has an amino acid sequence substantially identical to the polypeptide sequence described in Figure 3. Another preferred M3 polypeptide is a recombinant polypeptide capable of mediating immunomodulatory events wherein the polypeptide includes a domain having a sequence which has about 80% identity to SEQ ID NO:3. More preferably, the region of identity is 90% or greater; most preferably the region of identity is 95% or greater. "M3 polypeptide" further includes a polypeptide with about 50%, preferably about 75%, more preferably about 90%, and most preferably at least 95% of the biological activity, *e.g.*, immunomodulatory or anti-inflammatory activity, compared to a reference M3 polypeptide having an amino acid sequence substantially identical to SEQ ID NO:3. The term M3 polypeptide also includes homologs, e.g. human or murine homologs, of the sequences described herein. Homologs of the M3 protein may be derived from different strains of γHV68 virus or

other gammaherpesviruses, including, but not limited to, bovine gammaherpes viruses (*e.g.*, alcelaphine herpesvirus type 1, ovine herpesvirus type 2 and bovine lymphotropic herpesvirus) and porcine gammaherpes viruses (*e.g.*, porcine lymphotropic herepesvirus type 1 and type 2).

By "biologically active fragment" is meant a polypeptide fragment of a M3 polypeptide that exhibits immunomodulatory properties that are at least 30%, preferably at least 50%, more preferably at least 75%, and most preferably at least 100%, compared with the immunomodulatory properties of a full length M3 polypeptide. By "analog" is meant any substitution, addition, or deletion in the amino acid sequence of a M3 polypeptide that exhibits properties that are at least 30%, preferably at least 50%, more preferably at least 75%, and most preferably at least 100%, compared with the immunomodulatory properties of a M3 virus polypeptide from which it is derived. Fragments and analogs can be generated using standard techniques, for example, solid phase peptide synthesis or polymerase chain reaction.

"M3 immunomodulatory nucleic acid molecule" or "M3 nucleic acid molecule" means a nucleic acid molecule a nucleic acid molecule, such as a genomic DNA, cDNA, or RNA (*e.g.*, mRNA) molecule, that encodes a polypeptide having the characteristics or biological activities of any M3 polypeptide described herein, or a fragment thereof. A M3 nucleic acid molecule includes a nucleic acid having about 50% nucleic acid sequence identity, preferably about 75% nucleic acid sequence identity, more preferably about 90% nucleic acid sequence identity, and most preferably about 95% nucleic acid sequence identity to a reference M3 nucleic acid molecule as described herein. "M3 nucleic acid molecule" also includes a nucleic acid having about 50%, preferably about 75%, more preferably about 90%, and most preferably at least 95% of the function of a reference M3 nucleic acid having a sequence substantially identical to SEQ ID NO:1 or SEQ ID NO:2. Nucleic acid molecules that result from conservative substitutions in SEQ ID NO:1 or SEQ ID NO:2 are also encompassed by the term M3 nucleic acid molecule.

The term "identity" is used herein to describe the relationship of the sequence of a particular nucleic acid molecule or polypeptide to the sequence of a reference molecule of the same type. For example, if a polypeptide or nucleic acid molecule has the same amino acid or nucleotide residue at a given position, compared to a reference molecule to which it is aligned, there is said to be "identity" at that position. The level of

sequence identity of a nucleic acid molecule or a polypeptide to a reference molecule is typically measured using sequence analysis software with the default parameters specified therein, such as the introduction of gaps to achieve an optimal alignment (*e.g.*, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705, BLAST, or PILEUP/PRETTYBOX programs). These software programs match identical or similar sequences by assigning degrees of identity to various substitutions, deletions, or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine, valine, isoleucine, and leucine; aspartic acid, glutamic acid, asparagine, and glutamine; serine and threonine; lysine and arginine; and phenylalanine and tyrosine.

A nucleic acid molecule or polypeptide is said to be "substantially identical" to a reference molecule if it exhibits, over its entire length, at least 50%, preferably at least 55%, 60%, or 65%, and most preferably 75%, 85%, 90%, 95%, or 99% identity to the sequence of the reference molecule. For polypeptides, the length of comparison sequences is at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably at least 35 amino acids. For nucleic acid molecules, the length of comparison sequences is at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably at least 110 nucleotides.

Alternatively, or additionally, two nucleic acid sequences are "substantially identical" if they hybridize under high stringency conditions. By "high stringency conditions" is meant conditions that allow hybridization comparable with the hybridization that occurs using a DNA probe of at least 500 nucleotides in length, in a buffer containing 0.5 M $NaHPO_4$, pH 7.2, 7% SDS, 1 mM EDTA, and 1% BSA (fraction V), at a temperature of 65 C, or a buffer containing 48% formamide, 4.8X SSC, 0.2 M Tris-Cl, pH 7.6, 1X Denhardt's solution, 10% dextran sulfate, and 0.1% SDS, at a temperature of 42 C. (These are typical conditions for high stringency northern or Southern hybridizations.) High stringency hybridization is also relied upon for the success of numerous techniques routinely performed by molecular biologists, such as high stringency PCR, DNA sequencing, single strand conformational polymorphism analysis, and *in situ* hybridization. In contrast to northern and Southern hybridizations, these techniques are usually performed with relatively short probes (*e.g.*, usually 16

nucleotides or longer for PCR or sequencing and 40 nucleotides or longer for *in situ* hybridization). The high stringency conditions used in these techniques are well known to those skilled in the art of molecular biology, and examples of them can be found, for example, in Ausubel *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY, 1998, which is hereby incorporated by reference.

By "probe" or "primer" is meant a single-stranded DNA or RNA molecule of defined sequence that can base pair to a second DNA or RNA molecule that contains a complementary sequence ("target"). The stability of the resulting hybrid depends upon the extent of the base pairing that occurs. This stability is affected by parameters such as the degree of complementarity between the probe and target molecule, and the degree of stringency of the hybridization conditions. The degree of hybridization stringency is affected by parameters such as the temperature, salt concentration, and concentration of organic molecules, such as formamide, and is determined by methods that are well known to those skilled in the art. Probes or primers specific for M3 nucleic acid molecules, preferably, have greater than 45% sequence identity, more preferably at least 55-75% sequence identity, still more preferably at least 75-85% sequence identity, yet more preferably at least 85-99% sequence identity, and most preferably 100% sequence identity to the nucleic acid sequences encoding the amino acid sequences described herein. Probes can be detectably-labeled, either radioactively or non-radioactively, by methods that are well-known to those skilled in the art. Probes can be used for methods involving nucleic acid hybridization, such as nucleic acid sequencing, nucleic acid amplification by the polymerase chain reaction, single stranded conformational polymorphism (SSCP) analysis, restriction fragment polymorphism (RFLP) analysis, Southern hybridization, northern hybridization, *in situ* hybridization, electrophoretic mobility shift assay (EMSA), and other methods that are well known to those skilled in the art. A molecule, *e.g.*, an oligonucleotide probe or primer, a gene or fragment thereof, a cDNA molecule, a polypeptide, or an antibody, can be said to be "detectably-labeled" if it is marked in such a way that its presence can be directly identified in a sample. Methods for detectably-labeling molecules are well known in the art and include, without limitation, radioactive labeling (*e.g.*, with an isotope, such as ^{32}P or ^{35}S) and nonradioactive labeling (*e.g.*, with a fluorescent label, such as fluorescein).

By a "substantially pure polypeptide" is meant a polypeptide (or a fragment thereof) that has been separated from proteins and organic molecules that naturally

accompany it. Typically, a polypeptide is substantially pure when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the polypeptide is a M3 polypeptide that is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, pure. A substantially pure M3 polypeptide can be obtained, for example, by extraction from a natural source (*e.g.*, a mammalian cell), by expression of a recombinant nucleic acid molecule encoding a M3 polypeptide, or by chemical synthesis. Purity can be measured by any appropriate method, *e.g.*, by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

A polypeptide is substantially free of naturally associated components when it is separated from those proteins and organic molecules that accompany it in its natural state. Thus, a protein that is chemically synthesized or produced in a cellular system different from the cell in which it is naturally produced is substantially free from its naturally associated components. Accordingly, substantially pure polypeptides not only include those derived from eukaryotic organisms, but also those synthesized in *E. coli* or other prokaryotes.

An antibody is said to "specifically bind" to a polypeptide if it recognizes and binds to the polypeptide (*e.g.*, a M3 polypeptide), but does not substantially recognize and bind to other molecules (*e.g.*, non-M3 related polypeptides) in a sample, *e.g.*, a biological sample, that naturally includes the polypeptide. A preferred antibody binds to any M3 polypeptide sequence that is substantially identical to the polypeptide sequence shown in Figure 3, or portions thereof.

"Substantially pure nucleic acid molecule" means a nucleic acid molecule that is free of the components that naturally accompany it. For example, a substantially pure DNA is free of the genes that, in the naturally-occurring genome of the organism from which the DNA of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or that exists as a separate molecule (*e.g.*, a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA that is part of a hybrid gene encoding additional polypeptide sequence.

By a "promoter" is meant a minimal nucleic acid sequence element sufficient to direct transcription. If desired, constructs of the invention can include promoter elements that are sufficient to render promoter-dependent gene expression controllable in a cell type-specific, tissue-specific, or temporal-specific manner, or inducible by external signals or agents. Such elements can be located in the 5', 3', or intron regions of a gene.

By "operably linked" is meant that a gene and one or more regulatory sequences are connected in such a way as to permit gene expression when the appropriate molecules (*e.g.*, transcriptional activator proteins) are bound to the regulatory sequences.

By a "transgene" is meant a DNA molecule that is inserted by artifice into a cell (*e.g.*, the nuclear genome of a cell), and is incorporated into the genome of an organism that develops from the cell. Such a transgene can be partly or entirely heterologous (*i.e.*, foreign) to the transgenic organism, or can be a gene that is homologous to an endogenous gene of the organism. An organism or animal (*e.g.*, a mammal, such as a mouse, rat, or goat) can be said to be "transgenic" if it developed from a cell that had a transgene inserted into it by artifice.

By a "knockout mutation" is meant an artificially-induced alteration in a nucleic acid molecule (created by recombinant DNA technology or deliberate exposure to a mutagen) that reduces the biological activity of the polypeptide normally encoded therefrom by at least 80% relative to the unmutated gene. The mutation can be, without limitation, an insertion, deletion, frameshift mutation, or a missense mutation. A "knockout animal" is preferably a mammal, and more preferably a mouse, containing a knockout mutation, as defined above.

By "vector" is meant a genetically engineered plasmid or virus, derived from, for example, a bacteriophage, adenovirus, retrovirus, poxvirus, herpesvirus, or artificial chromosome, that is used to transfer a polypeptide (*e.g.*, a M3 polypeptide) coding sequence, operably linked to a promoter, into a host cell, such that the encoded peptide or polypeptide is expressed within the host cell.

"Conserved region" means any stretch of six or more contiguous amino acids exhibiting at least 80%, preferably 90%, and most preferably 95% amino acid sequence identity between two or more of the M3 family members.

By "transformation" is meant any method for introducing foreign molecules (*e.g.*, nucleic acid molecules) into a cell. Lipofection, DEAE-dextran-mediated

transfection, microinjection, protoplast fusion, calcium phosphate precipitation, retroviral delivery, electroporation, and biolistic transformation are just a few of the many transformation methods that are well known to those skilled in the art that can be used in the invention. For example, biolistic transformation is a method for introducing foreign molecules into a cell using velocity-driven microprojectiles such as tungsten or gold particles. Such methods can include helium-driven, air-driven, and gunpowder-driven techniques. Biolistic transformation can be applied to the transformation or transfection of a wide variety of cell types, intracellular organelles, and intact tissues including, without limitation, mitochondria, chloroplasts, bacteria, yeast, fungi, algae, animal tissue, and cultured cells. A "transformed cell," "transfected cell," or "transduced cell," is a cell (or a descendent of a cell) into which a DNA molecule encoding a polypeptide has been introduced, by means of recombinant DNA techniques.

By "sample" is meant a tissue biopsy, amniotic fluid, cell, blood, serum, urine, stool, or other specimen obtained from a patient or test subject. The sample can be analyzed to detect a mutation in a M3 gene, expression levels of a M3 gene or polypeptide, or the biological function of a M3 polypeptide, by methods that are known in the art. For example, methods such as sequencing, single-strand conformational polymorphism (SSCP) analysis, or restriction fragment length polymorphism (RFLP) analysis of PCR products derived from a patient sample can be used to detect a mutation in a M3 gene; ELISA can be used to measure levels of M3 polypeptide; and PCR can be used to measure the level of an M3 nucleic acid molecule.

By "neutralizing antibody" is meant an antibody that interferes with any of the biological activities of a M3 polypeptide, (*e.g.*, the ability of the M3 polypeptide M3 to inhibit T cell costimulatory signals). A neutralizing antibody may reduce the ability of a M3 polypeptide to carry out its specific biological activity by about 50%, more preferably by about 70%, and most preferably by about 90% or more. Any standard assay for the biological activity of any M3 polypeptide, including those described herein, may be used to assess potentially neutralizing antibodies that are specific for M3 polypeptides.

By "pharmaceutically acceptable carrier" is meant a carrier that is physiologically acceptable to the treated mammal while retaining the therapeutic properties of the compound with which it is administered. One exemplary pharmaceutically acceptable carrier is physiological saline solution. Other

physiologically acceptable carriers and their formulations are known to one skilled in the art and described, for example, in *Remington: The Science and Practice of Pharmacy*, (19th edition), ed. A. Gennaro, 1995, Mack Publishing Company, Easton, PA.

By "test compound" is meant a chemical, be it naturally-occurring or artificially-derived, that is assayed for its ability to act as an immunomodulator, an anti-inflammatory, or an anti-tumor agent by employing one of the assay methods described herein. Test compounds may include, for example, peptides, polypeptides, synthesized organic molecules, naturally occurring organic molecules, nucleic acid molecules, and components thereof.

"Therapeutically effective amount" as used herein in reference to dosage of a medication, refers to the administration of a specific amount of a pharmacologically active agent (*e.g.*, a M3 polypeptide) tailored to each individual patient manifesting symptoms characteristic of a specific disorder. For example, a patient receiving the treatment of the present invention might be experiencing an autoimmune or inflammatory disease. A person skilled in the art will recognize that the optimal dose of a pharmaceutical agent to be administered will vary from one individual to another. Dosage in individual patients should take into account the patients height, weight, rate of absorption and metabolism of the medication in question, the stage of the disorder to be treated, and what other pharmacological agents are administered concurrently.

By "treating " or "treatment" is meant the medical management of a patient with the intent to cure, ameliorate, stabilize, or prevent a disease, pathological condition, or disorder. This term includes active treatment, that is, treatment directed specifically toward the improvement or associated with the cure of a disease, pathological condition, or disorder, and also includes causal treatment, that is, treatment directed toward removal of the cause of the associated disease, pathological condition, or disorder. In addition, this term includes palliative treatment, that is, treatment designed for the relief of symptoms rather than the curing of the disease, pathological condition, or disorder; preventative treatment, that is, treatment directed to minimizing or partially or completely inhibiting the development of the associated disease, pathological condition, or disorder; and supportive treatment, that is, treatment employed to supplement another specific therapy directed toward the improvement of the associated disease, pathological

condition, or disorder. The phrase "treatment" also includes symptomatic treatment, that is, treatment directed toward constitutional symptoms of the associated disease, pathological condition, or disorder.

By "modulate" or "modulating" is meant changing, either by decrease or increase, the biological activity.

"Immune function" or "immunoreactivity" refers to the ability of the immune system to respond to foreign antigen as measured by standard assays well known in the art.

"Immunomodulation" or "immunomodulatory" refers to an alteration in the overall immunoreactivity of the immune system in a mammal upon treatment with an agent, such as a polypeptide of the present invention, or fragments and analogs thereof. "Immunomodulator" refers to an agent that induces an alteration (*i.e.*, immunosuppression or immunostimulation) as measured by an alteration of virulence in mutated viruses or a variety of immunoassays well known in the art (for example chemotaxis assays as described herein). For example, in the present invention, an immunomodulator may elicit an altered level of immune function whereby the alteration in the level of immune function identifies a M3 polypeptide. Preferably, the alteration is by at least 20-40%, more preferably by at least 50-75%, and most preferably by more than 80% relative to an untreated control of similar type. By "immunomodulatory disorder" is meant any pathophysiological condition that is characterized by an alteration in immune function. The alteration may include, for example, a decrease in immune cell number or size, an increase in cell apoptosis or death, or a decrease in immune cell growth, survival or differentiation. By "immunomodulatory disorder" is also meant any disease which involves the immune response or immunity in general. More specifically, such a disorder is a malfunction of the immune system that reduces the ability of an organism to resist foreign substances in the body (*e.g.*, viruses, bacteria, bacterial toxins, plant pollen, fungal spores, animal danders, medications, foods, allogeneic or xenogeneic transplanted organs) or causes the body to produce antibodies against its own tissues (*e.g.*, autoimmune disorders), resulting in tissue injury. Immunological disorders can also occur when a malfunctioning immune system (caused by, for example, genetic defect, illness, injury, malnutrition, medications such as those used for chemotherapy) results in an increase in frequency or severity of infections. Immunological disorders are often accompanied by inflammation, which is the body's

reaction to tissue injury, and results in the accumulation of white blood cells, macrophages, and lymphocytes at the site of injury.

By "pathophysiological condition" is meant a disturbance of function and/or structure of a living organism, resulting from an external source, a genetic predisposition, a physical or chemical trauma, or a combination of the above, including, but not limited to, any mammalian disease.

"Immunosuppression" refers to a decrease in the overall immunoreactivity of the immune system upon administration of an immunomodulator in comparison to the immunoreactivity of an immune system that has not been contacted with the particular immunomodulator. Preferably, the decrease is by at least 20-40%, more preferably by at least 50-75%, and most preferably by more than 80% relative to an untreated control of similar type.

"Immunostimulation" refers to a increase in the overall immunoreactivity of the immune system upon administration of an immunomodulator in comparison to the immunoreactivity of an immune system that has not been contacted with the particular immunomodulator. Preferably, the increase is by at least 20-40%, more preferably by at least 50-75%, and most preferably by more than 80%.

"Decreasing T cell stimulation" means lowering the level of T cell stimulation as measured by, for example, chromium release assay, by at least 20-40%, more preferably by at least 50-75%, and most preferably by more than 80% relative to an untreated control of similar type.

"Decreasing inflammation" means decreasing the number of inflammatory cells (leukocytes, for example eosinophils) in the target tissue by at least 20-40%, more preferably by at least 50-75%, and most preferably by more than 80% relative to an untreated control tissue of similar type. Preferably the decrease in the number of leukocyte cells is at least two-fold.

By "cell proliferation" is meant the growth or reproduction of similar cells. By "inhibiting proliferation" is meant the decrease in the number of similar cells by at least 10%, more preferably by at least 20%, and most preferably by at least 50%. By "stimulating proliferation" is meant an increase in the number of similar cells by at least 10%, more preferably by at least 20%, and most preferably by at least 50%.

By "apoptosis" is meant the process of cell death where a dying cell displays a set of well-characterized biochemical hallmarks which include cytolemmal blebbing, cell soma shrinkage, chromatin condensation, and DNA laddering.

An "anti-inflammatory" agent is an immunomodulatory agent capable of decreasing the overall inflammation or immune function upon administration to an individual. Preferably, the decrease is by at least 20-40%, more preferably by at least 50-75%, and most preferably by more than 80% relative to an untreated control of similar type.

By "cytokine" is meant a small molecular weight polypeptide that plays an important role in regulating the immune response, by for example, signaling adjacent cells.

By "chemokine" is meant a small molecular weight ligand which is a chemoattractant for leukocytes (*e.g.*, neutrophils, basophils, monocytes, and T cells), and is important for infiltration of lymphocytes and monocytes into sites of inflammation. The term "chemokine" refers to all known chemotactic cytokines expressed within mammalian organisms that mediate the recruitment and infiltration of leukocytes into tissues. The term "chemokine" includes but is not limited to all mammalian members of the C, CC, CXC, and CX_3C families of chemotactic cytokines, classified within the art based upon the distribution of cysteine residues therein.

"Chemokine receptor" refers to all known transmembrane proteins known within the art to interact with one or more chemokines. The term "chemokine receptor" shall include but is not limited to all chemokine receptors classified within the art as CR, CCR, CXCR and CXXXCR.

"Identifiable signal sequence" means a sequence of amino acids that may be identified by homology or biological activity to a peptide sequence with the known function of targeting a polypeptide to a particular region of the cell. Preferably the signal sequence directs the polypeptide to the cellular membrane wherefrom the polypeptide may be secreted. Alternatively, the signal sequence may direct the polypeptide to an intracellular compartment or organelle, such as the Golgi apparatus. One of ordinary skill in the art can identify a signal sequence by, for example, using readily available software (*e.g.*, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705, BLAST, or PILEUP/PRETTYBOX programs).

A "multi-transmembrane receptor protein" is an amphipathic protein having multiple hydrophobic regions that span the lipid bilayer of the cell membrane from the cytoplasm to the cell surface interspersed between hydrophilic regions that are exposed to water on both sides of the membrane. The number of hydrophobic regions in an amphipathic protein is often proportional to the number of times that proteins spans the lipid bilayer. Many multi-transmembrane receptor-related proteins act as receptors for various ligands (*e.g.*, cytokines) that act to initiate transduction of a signal from the cell surface where the ligand is binding to the interior of the cell. The initiation is often in the form of a conformational change in the multi-transmembrane receptor upon ligand binding. One of ordinary skill in the art can such protein by, for example, using readily available software (*e.g.*, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705, BLAST, or PILEUP/PRETTYBOX programs).

The following standard abbreviations are utilized throughout specification of the present invention and its included drawings: DNA - deoxyribonucleic acid; RNA - ribonucleic acid; C - cytosine; G - guanine; A - adenosine; T- thymidine; N - unknown; A, Ala - alanine; C, Cys - cysteine; D, Asp - aspartic acid; E, Glu - glutamic acid; F, Phe - phenylalanine; G, Gly - glycine; H, His - histidine; I, Ile - isoleucine; K, Lys - lysine; L, Leu - leucine; M, Met - methionine; N, Asn - asparagine; P, Pro - proline; Q, Gln - glutamine; R, Arg - arginine; S, Ser - serine; T, Thr - threonine; V, Val - valine; W, Trp - tryptophan; Y,Tyr - tyrosine; and pY, pTyr - phosphotyrosine.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be further understood and supported by reference to the following drawings and their associated descriptions. These descriptions and drawings are not meant to limit the invention and are to be interpreted as embodiments of the invention disclosed herein. The methods utilized in the generation of the data represented by these drawings are commonly known within the art and may be reproduced identically by employing the methods described herein.

Fig. 1 illustrates a genomic nucleic acid sequence of the M3 gene within γHV68

Fig. 2 illustrates a cDNA nucleic acid sequence which encodes an M3 polypeptide

Fig. 3 illustrates an amino acid sequence of M3 polypeptide expressed by γHV68

Fig. 4 illustrates the effect of M3 polypeptide in reducing atherosclerotic plaque deposition in a model of coronary restenosis after balloon angioplasty.

Figs. 5A and, 5B and 5C illustrates chemokine binding properties of an M3 polypeptide of the invention. Specifically, Fig. 5A illustrates competitive inhibition of ^{125}I-RANTES binding to an M3 polypeptide by CC chemokines. The percentage of maximal binding (mean and standard error of the mean) refers to binding in the absence of competitor (average value of maximal binding, 110,000 cpm). The K_d values determined are indicated. Fig. 5B illustrates competitive inhibition of ^{125}I-RANTES binding to the M3 protein by C and CX_3C chemokines. Fig. 5C illustrates competitive inhibition of ^{125}I-RANTES binding to the M3 protein by CXC chemokines.

DETAILED DESCRIPTION OF THE INVENTION

Typical naturally-occurring viral proteins act primarily as immunosuppressors. Conceivably, these molecules have been obtained by the virus through an ancient act of molecular piracy and subsequently are structurally optimized for a particular pharmacological phenotype of benefit to the virus. Against the extensive array of immune modalities, viruses have successfully co-evolved distinct strategies to thwart the host immune response. Like most viruses, gammaherpesvirus-68 has adapted to this inhospitable immune environment by expressing a wide range of proteins to systematically block each threatening aspect of the immune system.

The present invention provides a novel gene that has the potential to treat a wide range of immunopathological conditions. The present invention also provides novel therapeutic usage of polypeptides encoded within murine gammaherpesvirus-68 ("γHV68"). In particular, this invention embodies therapeutic utility of the polypeptide encoded by the third open reading frame within γHV68 (herein referred to as "M3"). The M3 protein is encoded between nucleotides 6060 and 7277 within the γHV68 genome. The genomic DNA sequence of M3 is herein displayed within Figure 1 as SEQ ID NO:1. A cDNA sequence which encodes an M3 polypeptide is herein displayed

within Figure 2 as SEQ ID NO:2. The amino acid sequence of M3 is herein displayed within Figure 3 as SEQ ID NO:3.

The functions of the protein encoded by this novel gene may range among a wide variety of possibilities. The gene may encode proteins that are important in modulating inflammation, or proteins that control viral replication and assembly. Alternatively, analysis of this gene and the polypeptides that it encodes may elucidate the mechanism utilized by M3 during infection and persistence within mammals. Still other proteins might be key regulators of cell proliferation or apoptosis.

One of ordinary skill in the art will readily appreciate that any such proteins might be useful as therapeutic agents to treat a variety of human diseases. For example, the present invention may yield novel anti-inflammatories (*e.g.*, for treatment of ischemic injury). Alternatively or additionally, the present invention may yield anti-viral compounds (*e.g.*, for treatment of AIDS). Yet other proteins may be used as drugs for treatment of proliferative diseases (*e.g.*, cancer or myelodysplastic syndrome) or cell death (*e.g.*, neurodegeneration, muscular dystrophy, cirrhosis of the liver).

M3 Protein Expression

In general, M3 proteins according to the invention, may be produced by transformation of a suitable host cell with all or part of a M3 protein-encoding cDNA fragment (*e.g.*, the cDNAs described above) in a suitable expression vehicle.

Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems may be used to provide the recombinant protein. The precise host cell used is not critical to the invention. The M3 protein may be produced in a prokaryotic host (*e.g.*, *E. coli*) or in a eukaryotic host (*e.g.*, Saccharomyces cerevisiae, insect cells, *e.g.*, Sf21 cells, or mammalian cells, *e.g.*, COS 1, NIH 3T3, or HeLa cells). Such cells are available from a wide range of sources (*e.g.*, the American Type Culture Collection, Rockland, MD; also, see, *e.g.*, Ausubel *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1994). The method of transformation or transfection and the choice of expression vehicle will depend on the host system selected. Transformation and transfection methods are described, *e.g.*, in Ausubel *et al.* (*supra*); expression vehicles may be chosen from those provided, *e.g.*, in *Cloning Vectors: A Laboratory Manual*, P.H. Pouwels *et al.*, 1985, Supp. 1987).

One preferred expression system is the baculovirus system (using, for example, the vector pBacPAK9) available from Clontech (Pal Alto, CA). If desired, this system

may be used in conjunction with other protein expression techniques, for example, the myc tag approach described by Evan *et al.* (*Mol. Cell Biol.* 5:3610-3616, 1985).

Alternatively, a M3 protein is produced by a stably-transfected mammalian cell line. A number of vectors suitable for stable transfection of mammalian cells are available to the public, *e.g.*, see Pouwels *et al.* (*supra*); methods for constructing such cell lines are also publicly available, *e.g.*, in Ausubel *et al.* (*supra*). In one example, cDNA encoding the M3 protein is cloned into an expression vector which includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, the M3 protein-encoding gene into the host cell chromosome is selected for by inclusion of 0.01-300 M methotrexate in the cell culture medium (as described in Ausubel *et al.*, *supra*). This dominant selection can be accomplished in most cell types. Recombinant protein expression can be increased by DHFR-mediated amplification of the transfected gene. Methods for selecting cell lines bearing gene amplifications are described in Ausubel *et al.* (*supra*); such methods generally involve extended culture in medium containing gradually increasing levels of methotrexate. DHFR-containing expression vectors commonly used for this purpose include pCVSEII-DHFR and pAdD26SV(A) (described in Ausubel *et al.*, *supra*). Any of the host cells described above or, preferably, a DHFR-deficient CHO cell line (*e.g.*, CHO DHFR$^-$ cells, ATCC Accession No. CRL 9096) are among the host cells preferred for DHFR selection of a stably-transfected cell line or DHFR-mediated gene amplification.

Once the recombinant M3 protein is expressed, it is isolated, *e.g.*, using affinity chromatography. In one example, an anti-M3 protein antibody (*e.g.*, produced as described herein) may be attached to a column and used to isolate the M3 virus protein. Lysis and fractionation of M3 protein-harboring cells prior to affinity chromatography may be performed by standard methods (see, *e.g.*, Ausubel *et al.*, *supra*). In another example, M3 proteins may be purified or substantially purified from a mixture of compounds such as an extract or supernatant obtained from cells (Ausubel *et al.*, *supra*). Standard purification techniques can be used to progressively eliminate undesirable compounds from the mixture until a single compound or minimal number of effective compounds has been isolated.

Once isolated, the recombinant protein can, if desired, be further purified, *e.g.*, by high performance liquid chromatography (see, *e.g.*, Fisher, *Laboratory Techniques In Biochemistry And Molecular Biology*, eds., Work and Burdon, Elsevier, 1980).

Polypeptides of the invention, particularly short M3 protein fragments, can also be produced by chemical synthesis (*e.g.*, by the methods described in *Solid Phase Peptide Synthesis*, 2nd ed., 1984 The Pierce Chemical Co., Rockford, IL).

These general techniques of polypeptide expression and purification can also be used to produce and isolate useful M3 protein fragments or analogs (described herein).

In certain preferred embodiments, the M3 protein might have attached any one of a variety of tags. Tags can be amino acid tags or chemical tags and can be added for the purpose of purification (for example a 6-histidine tag for purification over a nickel column). In other preferred embodiments, various labels can be used as means for detecting binding of a M3 protein to another protein, for example to a chemokine or a chemokine receptor. Alternatively, M3 DNA or RNA may be labeled for detection, for example in a hybridization assay. M3 nucleic acids or proteins, or derivatives thereof, may be directly or indirectly labeled, for example, with a radioscope, a fluorescent compound, a bioluminescent compound, a chemiluminescent compound, a metal chelator or an enzyme. Those of ordinary skill in the art will know of other suitable labels or will be able to ascertain such, using routine experimentation. In yet another preferred embodiment of the invention, the proteins disclosed herein, or derivatives thereof, are linked to toxins. Proteins linked to toxins can be used, for example to target toxic drugs to malignant tumors if the protein has the ability localize to the tumor.

Anti-M3 Protein Antibodies

To generate M3 protein-specific antibodies, a M3 protein coding sequence (*i.e.*, mVOX-2) may be expressed, for example, as a C-terminal fusion with glutathione S-transferase (GST) (Smith *et al.*, *Gene* 67:31-40, 1988). The fusion protein may then be purified on glutathione-Sepharose beads, eluted with glutathione cleaved with thrombin (at the engineered cleavage site), and purified to the degree necessary for immunization of rabbits. Primary immunizations are carried out with Freud's complete adjuvant and subsequent immunizations with Freud's incomplete adjuvant. Antibody titres are monitored by Western blot and immunoprecipitation analyzes using the thrombin-cleaved M3 protein fragment of the GST-M3 fusion protein. Immune sera are affinity purified using CNBr-Sepharose-coupled M3 protein. Antiserum specificity is

determined using a panel of unrelated GST proteins (including GSTp53, Rb, HPV-16 E6, and E6-AP) and GST-trypsin (which was generated by PCR using known sequences).

As an alternate or adjunct immunogen to GST fusion proteins, peptides corresponding to relatively unique hydrophilic M3 proteins may be generated and coupled to keyhole limpet hemocyanin (KLH) through an introduced C-terminal lysine. Antiserum to each of these peptides is similarly affinity purified on peptides conjugated to BSA, and specificity tested in ELISA and Western blots using peptide conjugates, and by Western blot and immunoprecipitation using M3 protein expressed as a GST fusion protein.

Alternatively, monoclonal antibodies may be prepared using the M3 proteins described above and standard hybridoma technology (see, *e.g.*, Kohler *et al.*, *Nature*, 256:495, 1975; Kohler *et al.*, *Eur. J. Immunol.* 6:511, 1976; Kohler *et al.*, *Eur. J. Immunol.* 6:292, 1976; Hammerling *et al.*, In Monoclonal Antibodies and T Cell Hybridomas, Elsevier, NY, 1981; Ausubel *et al.*, supra). Once produced, monoclonal antibodies are also tested for specific M3 protein recognition by Western blot or immunoprecipitation analysis (by the methods described in Ausubel *et al.*, supra). Antibodies which specifically recognize M3 proteins are considered to be useful in the invention; such antibodies may be used, *e.g.*, in an immunoassay to monitor the level of M3 proteins produced by a mammal (for example, to determine the amount or location of a M3 protein).

Preferably, antibodies of the invention are not only produced using the whole M3 polypeptide, but using fragments of the M3 polypeptide which lie outside highly conserved regions and appear likely to be antigenic, by criteria such as high frequency of charged residues may also be used. In one specific example, such fragments are generated by standard techniques of PCR and cloned into the pGEX expression vector (Ausubel *et al.*, *supra*). Fusion proteins are expressed in *E. coli* and purified using a glutathione agarose affinity matrix as described in Ausubel *et al.* (*supra*). To attempt to minimize the potential problems of low affinity or specificity of antisera, two or three such fusions are generated for each protein, and each fusion is injected into at least two rabbits. Antisera are raised by injections in a series, preferably including at least three booster injections.

Identification of Molecules that Modulate M3 Biological Activity or Whose Biological Activity is Modulated by M3

Isolation of the M3 cDNA (as described herein) also facilitates the identification of molecules that increase or decrease a M3 polypeptide biological activity. Similarly, molecules whose activity is modulated by a M3 polypeptide biological activity can be identified. According to one approach, candidate molecules are added at varying concentrations to the culture medium of cells expressing M3 mRNA. M3 polypeptide biological activity is then measured using standard techniques. The measurement of biological activity can include the measurement of M3 polypeptide protein and nucleic acid molecule levels, or the effect of M3 polypeptide on immunomodulation

If desired, the effect of candidate modulators on expression can, in the alternative, be measured at the level of M3 protein production using the same general approach and standard immunological detection techniques, such as western blotting or immunoprecipitation with a M3 polypeptide-specific antibody (see below).

Candidate modulators can be purified (or substantially purified) molecules or can be one component of a mixture of compounds (*e.g.*, an extract or supernatant obtained from cells; Ausubel *et al.*, *supra*). In a mixed compound assay, M3 polypeptide expression is tested against progressively smaller subsets of the candidate compound pool (*e.g.*, produced by standard purification techniques, *e.g.*, HPLC or FPLC) until a single compound or minimal compound mixture is demonstrated to modulate M3 polypeptide expression.

Alternatively, or in addition, candidate compounds can be screened for those that modulate M3 polypeptide activity. In this approach, the level of immunomodulation in the presence of a candidate compound is compared to the level of immunomodulation in its absence, under equivalent conditions. Again, such a screen can begin with a pool of candidate compounds, from which one or more useful modulator compounds is isolated in a step-wise fashion.

The screening assays described above can be carried out in a variety of ways that are well known to those skilled in this art. These include using M3 polypeptide variants or by using fragments of a M3 polypeptide.

A test compound that can be screened in the methods described above can be a chemical, be it naturally-occurring or artificially-derived. Such compounds can include, for example, polypeptides, synthesized organic molecules, naturally occurring organic

molecules, nucleic acid molecules, and components thereof. Candidate M3 polypeptide modulators include peptide as well as non-peptide molecules (*e.g.*, peptide or non-peptide molecules found, *e.g.*, in a cell extract, mammalian serum, or growth medium in which mammalian cells have been cultured).

In general, novel drugs for prevention or treatment of immunomodulatory diseases are identified from large libraries of both natural products, synthetic (or semi-synthetic) extracts, and chemical libraries using methods that are well known in the art. Those skilled in the field of drug discovery and development will understand that the precise source of test extracts or compounds is not critical to the screening methods of the invention. Accordingly, virtually any number of chemical extracts or compounds can be screened using these methods. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic-, or animal-based extracts, fermentation broths, and synthetic compounds, as well as modifications of existing compounds.

Numerous methods are also available for generating random or directed synthesis (*e.g.*, semi-synthesis or total synthesis) of any number of chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, and nucleic acid molecule-based compounds. Synthetic compound libraries are commercially available from Brandon Associates (Merrimack, NH) and Aldrich Chemical (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are commercially available from a number of sources, including Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch Oceanographic Institute (Ft. Pierce, FL), and PharmaMar, U.S.A. (Cambridge, MA). In addition, natural and synthetically produced libraries are produced, if desired, according to methods known in the art, *e.g.*, by standard extraction and fractionation methods. Furthermore, if desired, any library or compound can be readily modified using standard chemical, physical, or biochemical methods.

In addition, those skilled in the art of drug discovery and development readily understand that methods for dereplication (*e.g.*, taxonomic dereplication, biological dereplication, and chemical dereplication, or any combination thereof) or the elimination of replicates or repeats of materials already known for their therapeutic activities for immunological disorders can be employed whenever possible.

When a crude extract is found to regulate immunomodulation, further fractionation of the positive lead extract can be carried out to isolate chemical constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and identification of a chemical entity within the crude extract having a desired activity. The same assays described herein for the detection of activities in mixtures of compounds can be used to purify the active component and to test derivatives thereof. Methods of fractionation and purification of such heterogeneous extracts are known in the art. If desired, compounds shown to be useful agents for treatment are chemically modified according to methods known in the art. Compounds identified as being of therapeutic value can be subsequently analyzed using, for example, any of the animal models described herein.

Testing and Administration of Immunomodulatory Molecules

M3 immunomodulatory polypeptides for use in the methods and formulations of the present invention may be screened for immunomodulatory activity. For example, chemotaxis activity in the presence of a candidate compound is compared to chemotaxis activity in its absence, under equivalent conditions. Such a screen may begin with a pool of candidate compounds, from which one or more useful immunomodulator compounds are isolated in a step-wise fashion. Chemotaxis (of eosinophil or other leukocyte) activity may be measured by any standard assay, for example, those described herein.

Candidate M3 encoded modulators include peptide as well as non-peptide molecules (*e.g.*, peptide or non-peptide molecules found, *e.g.*, in a cell extract, mammalian serum, or growth medium on which mammalian cells have been cultured). Particularly useful are modulators of M3 protein expression.

A molecule which promotes an increase in M3 protein expression or a decrease in leukocyte chemotaxis activity is considered particularly useful in the invention; such a molecule may be used, for example, as a therapeutic to decrease the immunoreactivity in a individual. In addition, M3 proteins identified in the present invention (or modifications and derivatives thereof) that exhibit these activities are also particularly desirable. For example, these polypeptides may be used to treat an individual that has rheumatoid arthritis. Other human diseases that may be treated using a molecule that act as an immunosuppressant or otherwise reduces the immune function include, acute

inflammation, allergic reactions, asthmatic reactions, inflammatory bowel diseases (*i.e.*, Crohn's Disease and ulcerative colitis), transplant rejection, and restenosis.

A molecule which enhances or induces apoptosis may alternatively be used in the treatment of tumors.

Immunomodulators of this invention and other polypeptides found to be effective at the level of M3 protein expression or activity may be confirmed as useful in animal models. The animal models that may be used in the present invention will test drug candidates for efficacy in treating autoimmune and inflammatory diseases. Target therapeutic areas include acute inflammation, rheumatoid arthritis, transplant rejection, asthma, inflammatory bowel disease, uveitis, restenosis, multiple sclerosis, psoriasis, wound healing, lupus erythematosus and any other autoimmune or inflammatory disorder that can be recognized by one of ordinary skill in the art.

For example, other diseases related to inflammation that may be treated by methods the present invention include, for example, allergic rhinitis, atopic dermatitis and food allergies. Examples of other autoimmune disorders, where the immune system attacks the host's own tissues, include, but are not limited to type 1 insulin-dependent diabetes mellitus, deramatitis, meningitis, thrombotic thrombocytopenic purpura, Sjogren's syndrome, encephalitis, leukocyte adhesion deficiency, rheumatic fever, Reiter's syndrome, psoriatic arthritic, progressive systemic sclerosis, primary biliary cirrhosis, pemphigus, pemphigoid, necrotizing vasculitis, mayasthenia gravis, lupus erythmatosus, polymyositis, sarcoidosis, granulomatorsis, vasculitis, pernicious anemia, CNS inflammatory disorder antigen-antibody complex mediated diseases, autoimmune hemolytic anemia, Hashimoto's thyroiditis, Graves disease, habitual spontaneous abortions, Reynard's syndrome, glomerulonephritis, dermatomyositis, chromic active hepatitis, celiac disease, autoimmune complications of AIDS, atrophic gastritis, ankylosing spondylitis and Addison's disease.

Other diseases related to non-malignant or immunological-related cell-proliferative diseases that may be treated by methods the present invention include for example psoriasis, penphigus vularis, Behcet's syndrome, acute respiratory distress syndrome (ARDS), ischemic heart disease, atherosclerosis, post-dialysis syndrome, leukemia, acquired immune deficiency syndrome septic shock and other type of acute inflammation, and lipid histiocytosis.

If successful, the identified M3 polypeptide may be used as anti-inflammatory or anti-cancer therapeutics (*e.g.*, a mouse tumor model)

Animal models for testing the immunomodulatory effects of candidate compounds are well known in the art. Therefore, the present invention merely refers to a selection of animal models that can be used to test the candidate compounds of the invention and omits what is well known in the art. Animal models proposed for use in the present invention to test candidate compounds for their efficacy in treating autoimmune and inflammatory disorders include, but are not limited to:

Acute inflammation:

Animal models of acute inflammation are targeted for initial, fast drug efficacy screening and for their potential predicative value of outcomes in chronic inflammatory diseases. The following animal models may be used to test the candidate compounds of the present invention for their efficacy in treating acute inflammation: 1) carrageenin induced inflammation 2) turpentine induced inflammation 3) transgenic HLAB-27 inflammatory and 4) ear-scratch model of inflammation.

Rheumatoid arthritis; rat, mouse and rabbit

Efficacy in Rheumatoid Arthritis evaluated in 1) various antigen induced arthritis models in rabbit, rat and mouse; and 2) in transgenic rheumatological models.

The molecular and cellular mechanisms of action of the candidate compounds will be evaluated by testing their efficacy in influencing key intracellular mechanisms that regulate degradative processes involved in joint disease. Important molecular and cellular mechanisms that will receive particular focus include signaling events regulating disease processes such as increased angiogenesis, synovial hyperplasia and matrix metalloprotease expression. These processes are thought to be involved in cartilage degradation in arthritic diseases.

1. Collagen induced arthritis; rat, mouse and rabbit

Autoimmune-mediated polyarthritis can be induced in certain strains of rodents (rat, mouse and rabbit) and non-human primates by immunizing them with native type II collagen. The collagen induced arthritis model is widely used and well characterized. Collagen induced arthritis is mediated by susceptibility to autoantibodies which bind to a

particular region of type II collagen. The mechanism of induction is linked to MHC-class II molecules but also depends on the species of type II collagen used for immunization.

2. Ovalbumin induced arthritis; rabbit

Candidate compounds are tested for efficacy in decreasing signs and symptoms of ovalbumin arthritis. Polyarthritis is induced in rabbits by immunizing them with Ovalbumin.

3. Adjuvant induced arthritis; rat, mouse and rabbit

Candidate compounds are tested for the efficacy in decreasing signs and symptoms of adjuvant induced arthritis. Polyarthritis is induced in certain strains of rodents by immunizing them with Freud's Adjuvant.

4. Streptococcal cell wall-induced arthritis; rat

Candidate compounds are tested for efficacy in decreasing signs and symptoms of streptococcal cell wall-induced arthritis. Chronic, erosive polyarthritis is induced by intraperitoneal-injection of aqueous suspension of cell wall fragments, isolated from group A streptococci.

Transplant rejection (acute and chronic)

Efficacy in transplant rejection is evaluated in various models of graft vascular disease (GVD). GVD is the most common cause of late graft failure in solid organ transplantation. GVD or graft atherosclerosis is characterized by plaque formation and fibrosis in small vessels. The development of graft vascular disease has been associated with acute allograft rejection, ischemia-reperfusion injury and bacterial or viral infections. The common pathway of these postoperative insults results in perivascular inflammation which triggers migration of mesenchymal cells into the vessel wall eventually resulting in occlusion or partial occlusion of the vessel lumen.

1. *Aortic allograft model; rat, rabbit, monkey*

Candidate compounds are tested for efficacy in reducing graft atherosclerosis and transplant rejection in a model of vascular injury after transplantation of aortic segments performed in certain strains of MHC mismatched rats, and rabbits.

2. Tracheal allograft model; rat, rabbit, monkey

Candidate compounds are tested for efficacy in reducing graft atherosclerosis and transplant rejection in a model of vascular injury after transplantation of tracheal segments performed in certain strains of MHC mismatched rats, rabbits, and monkeys.

3. Heterotopic heart transplant; mouse, rat, monkey

A heterotropic heart transplantation is performed in MHC mismatched rats. In this model, rodents are treated with cyclosporine A for only the first 7 days after transplantation, are allowed to develop graft vascular disease, and are then analyzed after sacrifice at postoperative day 90.

4. Orthotopic kidney transplant; mouse, rat, monkey

An orthotopic kidney transplantation is performed in MHC mismatched rats. In this model, animals receiving subtherapeutic doses of cyclosporine A for the first 10 days after transplantation, are allowed to exhibit features of chronic renal allograft rejection in 70% of cases, and are then analyzed after sacrifice at postoperative day.

5. Orthotopic lung transplant; rat, monkey

Candidate compounds are tested for effectiveness in delaying or reducing signs and symptoms of organ rejection after lung whole organ transplantation in rats and monkeys.

6. Reperfusion injury; rat

The immediate postoperative course in clinical lung transplantation is often severely impaired by delayed graft function as a result of ischemia and reperfusion injury. Preventive efficacy of drug candidates in ischemia-

reperfusion injury is evaluated using a model of acute, *in vivo* double lung transplantation in the rat (Hausen *et al.*, *Ann Thorac Surg* 61:1714-9,1996 incorporated herein by reference).

Asthma; rodent

The effectiveness of candidate compounds in reduction of signs and symptoms of asthma is evaluated in rodent models of antigen induced experimental airways inflammation. The models include:

1. Ovalbumin induced experimental airways inflammation; rodent
   Candidate compounds are tested for efficacy in reducing inflammatory cell components in the bronchoalveolar lavage of the lungs after aerosol challenge in ovalbumin sensitized rodent models of experimental airways inflammation.

2. Ovalbumin induced allergic sensitization in presence of GM-CSF transgene expression; mice
   Candidate compounds are tested for efficacy in reducing inflammatory cell components in the bronchoalveolar lavage of the lungs of mice after ovalbumin aerosol challenge in the context of local expression of GM-CSF (Staempfli *et al.*, *J. Clin. Invest.*, Vol. 102:9, 1704-1714).

Inflammatory bowel disease (IBD); mice and rats

Drug candidates are evaluated for their potential therapeutic efficacy in ulcerative colitis or Crohn's disease utilizing various models of antigen induced and genetically mediated spontaneous chronic intestinal inflammation in mice and rats. Examples include:

1. Dextran sulfate sodium induced IBD; mice
   Chronic, irreversible clinical symptoms of IBD are induced by treating mice with an oral administration of dextran sulfate sodium.

2. Gene deletion and transgenic models for IBD; rodent

Compound efficacy will be tested in transgenic rodent lines which develop symptoms closely resembling the human elements of inflammatory bowel disease. Models include, the targeted deletion of the genes encoding IL-2, IL-10, TGF beta, T-cell receptor alpha/beta, keratin 8, Gi2 alpha. In addition, animals expressing transgenes for the human WA-B27 and HLA-B27 as well as a dominant negative construct which functionally blocks N-cadherin will be tested.

Uveitis

Drug candidates will be evaluated for efficacy in various animal models of uveitis. Key models include both, experimental autoimmune uveitis and adoptively transferred experimental autoimmune uveitis.

1. Experimental autoimmune uveitis (EAU)

EAU is a T-cell mediated inflammatory eye disease that can be induced in several mammalian species by immunization with ocular-specific antigens (Gery *et al.*, *Invest. Opthalmol. Vis. Sci.*, 27: 1296-1300, 1986,. Sanui *et al.*, *J. Exp. Med.*, 169:1947-1989, incorporated herein by reference). This experimental disease is considered a model for a family of inflammatory eye diseases in humans and has been used to examine numerous modalities before their human testing.

2. Adoptively transferred experimental autoimmune uveitis

Adoptively transferred EAU is induced through injection of lymphocytes presensitized against the retinal antigen are injected into naive syngenic recipients (McAllister *et al.*, *J. Immunol.*, 138:1416-1420, 1987 incorporated herein by reference)

Once identified, a M3 immunomodulator or anticarcinogen may be administered with a pharmaceutically-acceptable diluent, carrier, or excipient, at a pharmaceutically effective dose. Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer M3 proteins to patients suffering from presymptomatic carcinoma. Any appropriate route of administration may be

employed, for example, parenteral, intravenous, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, or oral administration. Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found in, for example, "Remington's Pharmaceutical Sciences." Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for NES1 modulatory compounds include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

If desired, treatment with a NES1 modulatory compound may be combined with more traditional cancer therapies such as surgery, radiation, or chemotherapy.

Detection of Specific Conditions

M3 polypeptides and nucleic acid sequences find diagnostic use in the detection or monitoring of inflammatory, autoimmune and other conditions. For example, because M3 proteins are involved in leukocyte chemotaxis and because a decrease in the number of leukocytes correlates with immunosuppression, an alteration in the level of particular M3 protein production provides an indication of the prognosis of the condition. Levels of M3 protein expression may be assayed by any standard technique. For example, its expression in a biological sample (*e.g.*, a biopsy) may be monitored by standard Northern blot analysis or may be aided by PCR (see, *e.g.*, Ausubel *et al.*, *supra; PCR Technology: Principles and Applications for DNA Amplification*, ed., H.A. Ehrlich, Stockton Press, NY; and Yap and McGee, Nucl. Acids. Res. 19:4294, 1991).

In yet another approach, immunoassays are used to detect or monitor M3 protein in a biological sample. M3 protein-specific polyclonal or monoclonal antibodies (produced as described above) may be used in any standard immunoassay format (*e.g.*, ELISA, Western blot, or RIA assay) to measure M3 polypeptide levels; again comparison is to wild-type M3 protein levels. A change in M3 protein production may be indicative of a particular prognosis. Examples of immunoassays are described, *e.g.*, in Ausubel *et al.*, *supra*. Immunohistochemical techniques may also be utilized for M3 protein detection. For example, a tissue sample may be obtained from a patient, and a section stained for the presence of M3 protein using an anti- M3 protein antibody and any standard detection system (*e.g.*, one which includes a secondary antibody conjugated to horseradish peroxidase). General guidance regarding such techniques can be found in, *e.g.*, Bancroft and Stevens (*Theory and Practice of Histological Techniques*, Churchill Livingstone, 1982) and Ausubel *et al.* (*supra*).

M3 Gene Therapy

Because expression of M3 protein may correlate with autoimmune, inflammatory or tumor prognosis, the M3 gene also finds use in immunomodulatory gene therapy. For example, to enhance leukocyte infiltration of a tumor, a functional M3 gene may be introduced into cells at the site of a tumor. In addition, M3 polypeptides that are shown to reverse autoimmune reactions may also be used in gene therapy. Alternatively, M3 polypeptides with alterations which block inflammatory may be administered via gene therapy for the treatment of eosinophil mediated inflammatory conditions.

Retroviral vectors, adenoviral vectors, adeno-associated viral vectors, or other viral vectors with the appropriate tropism for M3 protein-expressing cells may be used as a gene transfer delivery system for a therapeutic M3 gene construct. Numerous vectors useful for this purpose are generally known (Miller, *Human Gene Therapy* 15-14, 1990; Friedman, *Science* 244:1275-1281, 1989; Eglitis and Anderson, BioTechniques 6:608-614, 1988; Tolstoshev and Anderson, Current Opinion in Biotechnology 1:55-61, 1990; Sharp, *The Lancet* 337:1277-1278, 1991; Cornetta *et al.*, *Nucleic Acid Research and Molecular Biology* 36:311-322, 1987; Anderson, *Science* 226:401-409, 1984; Moen, Blood *Cells* 17:407-416, 1991; and Miller and Rosman, *Biotechniques* 7:980-990, 1989; Le Gal La Salle *et al.*, *Science* 259:988-990, 1993; and Johnson, Chest 107:77S-83S, 1995). Retroviral vectors are particularly well developed

and have been used in clinical settings (Rosenberg *et al.*, *N. Engl. J. Med* 323:370, 1990; Anderson *et al.*, U.S. Pat. No. 5,399,346).

Non-viral approaches may also be employed for the introduction of therapeutic DNA into cells. For example, M3 gene may be introduced into a tumor cell by the techniques of lipofection (Felgner *et al.*, *Proc. Natl. Acad. Sci.* USA 84:7413, 1987; Ono *et al.*, *Neuroscience Lett* 117:259, 1990; Brigham *et al.*, *Am. J. Med. Sci.* 298:278, 1989; Staubinger and Papahadjopoulos, *Meth. Enz.* 101:512, 1983); asialorosonucoid-polylysine conjugation (Wu and Wu, *J. Biol. Chem.* 263:14621, 1988; Wu *et al.*, *J. Biol. Chem.* 264:16985, 1989); or, less preferably, microinjection under surgical conditions (Wolff *et al.*, *Science* 247:1465, 1990).

For any of the above approaches, the therapeutic M3 DNA construct is preferably applied to the site of the malignancy or inflammation and cytotoxic damage (for example, by injection), but may also be applied to tissue in the vicinity of the malignancy or inflammation and cytotoxic damage or even to a blood vessel supplying these areas.

In the gene therapy constructs, M3 cDNA expression is directed from any suitable promoter (*e.g.*, the human cytomegalovirus, simian virus 40, or metallothionein promoters), and its production is regulated by any desired mammalian regulatory element. For example, if desired, enhancers known to direct preferential gene expression in endothelial or epithelial cells may be used to direct M3 protein expression. Such enhancers include, without limitation, the lung specific promotors (*e.g.* surfactant), and gut specific regulatory sequences.

Alternatively, if a M3 genomic clone is utilized as a therapeutic construct (for example, following its isolation by hybridization with the M3 cDNA described above), M3 protein expression is regulated by its cognate regulatory sequences or, if desired, by regulatory sequences derived from a heterologous source, *e.g.*, any of the promoters or regulatory elements described above.

Less preferably, M3 gene therapy is accomplished by direct administration of the M3 mRNA to a tumor. This mRNA may be produced and isolated by any standard technique, but is most readily produced by *in vitro* transcription using a M3 cDNA under the control of a high efficiency promoter (*e.g.*, the T7 promoter). Administration of M3 mRNA to malignant cells is carried out by any of the methods for direct nucleic acid administration described above.

Ideally, the production of M3 protein by any gene therapeutic approach described above results in a cellular level of M3 protein that is at least equivalent to the normal, cellular level of NES1 in an unaffected individual. Treatment by any NES1-mediated gene therapy approach may be combined with more traditional cancer therapies (*e.g.*, surgery, radiation, or chemotherapy for treatment of tumors).

Another therapeutic approach included within the invention involves direct administration of recombinant M3 protein, either to the site of a malignancy (for example, by injection) or systemically by any conventional recombinant protein administration technique for treatment of an autoimmune or inflammatory disorder. The actual dosage of M3 protein depends on a number of factors, including the size and health of the individual patient, but, generally, between 0.1 mg and 100 mg inclusive are administered per day to an adult in any pharmaceutically-acceptable formulation.

The above approaches may also be used to inhibit the activity of the candidate compound by substituting an altered M3 polypeptide having M3 protein blocking activity (*e.g.*, have a deletion or insertion at the amino terminus) for the M3 polypeptide described above.

Transgenic Animals

Transgenic animals may be made using standard techniques. For example, a M3 gene may be provided using endogenous control sequences or using constitutive, tissue-specific, or inducible regulatory sequences. Transgenic animals lacking functional M3 polypeptide may also be made using standard techniques. This may be done by engineering knock-out mutations in the M3 gene using DNA sequences provided herein.

M3 Gene Sequences

In general, the present invention relates to M3 nucleic acid. In some preferred embodiments the nucleic acid is genomic DNA. In other preferred embodiments the nucleic acid is cDNA. In yet other preferred embodiments, the nucleic acid is mRNA. In certain preferred aspects of the invention the M3 nucleic acid encodes an immunomodulatory polypeptide. Preferably the immunomodulatory polypeptide is M3 polypeptide. The immunomodulatory polypeptide may be an immunosuppressor or an immunostimulator. Preferably the invention relates to a polypeptide that is substantially identical (at least 80% identity) with the M3 polypeptide disclosed herein. For example,

the polypeptide may be any one, or a combination of a cytokine, an anti-inflammatory, an immunoreceptor, a multi-transmembrane receptor protein or a secreted protein.

M3 Knockouts

Herpesvirus proteins have been defined as virulence factors on the basis that, when present, they confer increased pathogenicity and improve viral replication within immunocompetent hosts. When genes that encode these virulence factor proteins are deleted, the resulting virus strain generally exhibits an attenuated or altered disease phenotype (Turner, Curr. Top. Microbiol. Immunol., 163:125-151, 1990; Buller Microbiol. Rev., 55:80-122, 1991; Smith, *J. Gen. Virol.*, 74:1725-1740; McFadden, Austin (TX): R. G. Landes Company, 1995, incorporated herein by reference). Such "knockout" herpesviruses may assist in the elucidation of the immunomodulatory or other role of viral proteins.

Using standard virological assays (Nash *et al.*, (1999) Immunological Review, 57:731 to end), one of ordinary skill in the art may establish the contribution of each gene to γHV68 infection and the general biochemical and physiological progression of infection. Knockout M3 lacking a particular gene may be generated using standard molecular biological techniques (see VAN Dyke *et al.*, J. Virol. 2000. 74(16): 7415 *et seq.* (incorporated herein by reference); Clambey *et al.*, J. Virol. 2000. 74(4): 1973-1984 (incorporated herein by reference); Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Press*, Cold Spring Harbor, NY, 1989; Innis *et al.*, PCR Protocols: *A Guide to Methods and Applications*, Academic Press, San Diego, CA, 1990; Erlich *et al.*, *PCR Technology: Principles and Applications for DNA Amplification*, Stockton Press, New York, NY, 1989 each of which is incorporated herein by reference). The virulence of these knockout viruses may be assessed using standard infectivity assays well known in the art (see Nash *et al.*, (1999) Immunological Review, 57:731 to end). Such a discovery may lead to a method of modulating T cell stimulation in humans.

Examples

Production of γHV68 infected cell supernatants. Murine 3T12 fibroblast cells were either mock infected or infected at a multiplicity of infection (MOI) of 5 with γHV68 virus (WUMS strain in DMEM supplemented with 10% fetal calf serum (FCS).

After one hour, the monolayer was washed with PBS, fresh DMEM containing 10% FCS was added, and infection was allowed to proceed for 8 hours at 37°C. The monolayer was then washed with PBS, fresh DMEM without FCS was added, and the infection was allowed to proceed for 20 hours at 37°C. The culture supernatant was passed through a 0.2 μM filter and concentrated 120 fold at 4°C (Centriprep-10, Amicon, Inc., Beverly, MA). Concentrated supernatants were centrifuged at 150,000 x g for 3 hrs to remove residual free virus and then stored at 4°C. The concentration of the M3 protein was determined by densitometry of silver-stained 12.5% acrylamide gels utilizing known amounts of purified bacterially expressed M3 protein (see below) as a standard. Densitometric comparison was performed with Kodak ID Image Analysis software (Eastman Kodak Company, Rochester, NY), and measurements were taken within the linear range of densitometrically determined ban intensities.

Cross-linking assay. The interaction of the M3 protein with various chemokines was detected using a chemical cross-linking assay described in Upton, C., Mossman, K. & McFadden, G. (1992) Science 258, 1369-1372. Briefly, γHV68 infected cell supernatants were incubated with the appropriate chemokine for 2 hours at room temperature. After incubation, the protein complexes were covalently cross linked with the addition of 1-ethyl-2-(3-dimethylaminopropyl) carbodiimide (EDC) (Sigma, St. Louis, MO) to a final concentration of 40 mM for 30 minutes at room temperature, and the reaction was quenched by the addition of 1/10 volume of 1. M Tris, pH7.5. Laemmli sample buffer containing 2-mercaptoethanol (sample buffer) was added to the mixtures, the samples were boiled for 3 minutes, separated on a 12% SDS-PAGE gel, and transferred to a nitrocellulose membrane for immunoblotting. M3 protein complexes were detected by probing with a 1:5,000 dilution of anti-M3 polyclonal rabbit antisera (Cocalico, Reamstown, PA), and a 1:5,000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G antibody.

Bacterial and baculovirus expression systems. The following primers were used to amplify the genomic region of γHV68 corresponding to the secreted form of the M3 protein, adding a NdeI site and six histidine residues at the 5' end, and a XhoI site at the 3' end 5'-ACATATGCACCATCATCATCATCATCCTACTCTAGGTTTGGCACCTGCT-3', and 5'-ACTCGAGTCTACTACTAATGATCCCCAAAATACTCCAGCCT-3'. The 1,187 bp fragment was sequenced and clonal into pet30a(+), and recombinant protein

was induced and purified over a nickel column as per the manufacturer's protocol (Novagen, Madison, WI). For baculovirus expression, the full length M3 open reading frame was amplified using the following primers: 5'-AGCGGCCGCATGGCCTTCCTATCCACATCTGTGCT-3' inserting a NotI site 5' of the M3 start methionine, and 5'-ACTCGAGTCTACTACTAATGATCCCCAAAATACTCCAGCCT-3', inserting a XhoI site 3' of the M3 stop condon. The 1,244 bp fragment was sequenced and cloned into the pFastBac vector, and recombinant baculovirus was generated following the Bact-to-Bac baculovirus expression system protocol (Life Technologies, www.lifetech.com). A control β-galactosidase expressing baculovirus was constructed at the same time using unmanipulated pFastBac vector containing the Lac Z gene. Supernatants from cells infected with M3 protein expressing and control baculoviruses were collected 4 days after infection of Sf9 cells, clarified by centrifugation (200 x g) for 5 minutes and then stored at 4°C.

Immunoprecipitation. Either 100 μL of γHV68 (M3 protein at ~3 μM) or mock infected 3T12 cell supernatants, or 100 μL of Sf9 cell supernatants after infection with either a M3 protein (M3 protein at ~500nM) or LacZ expressing baculovirus, were incubated with 500pM ^{125}I-labeled human RANTES or interleukin-5 (IL-5) (Amersham, Arlington Heights, IL) for 30 minutes at 4°C. Samples were first immunoprecipitated by incubation with 3μL of pre-immune rabbit sera for 60 minutes 4°C, followed by addition of 15 μL of protein A conjugated agarose beads (Calbiochem, La Jolla, CA) and incubation for an additional 120 minutes at 4°C. The beads were isolated by centrifugation for 15 minutes at 5000 rmp, washed three times with 500 μL phosphate buffered saline with 0.05% Tween-20, and resuspended in 20 μL of sample buffer. Supernatants were subsequently incubated with 5 μL of sera from a rabbit multiply immunized with bacterially expressed M3 protein, followed by incubation with 30μL of protein A conjugated agarose beads, recovery, and washing as above. Precipitated samples were resuspended in 20 μL of sample buffer, separated on a 20% acrylamide gel, and analyzed by autoradiography.

Binding assays. Saturation analysis determination of the disassociation constant for ^{125}I-labeled human RANTES binding to M3 protein was performed as follows: 250 pM M3 protein (as diluted γHV68 infected cell supernatants) was incubated with increasing amounts of ^{125}I-labeled human RANTES for 60 minutes. Bound RANTES

was recovered by incubation with 5 μL of α-M3 polyclonal sera, followed by incubation with 30 μL of protein A conjugated agarose beads, which were recovered, and washed as described above. Precipitated ^{125}I-RANTES was resuspended in 1mL scintillation fluid, and counts per minute were compared to the counts of input radioactivity to determine the amount of ^{125}RANTES bound. Competitive inhibition with CC and CXC chemokines was demonstrated by incubating 250 pM M3 protein with 500 pM of ^{125}I-RANTES and increasing amounts of unlabeled chemokine or IL-5, and precipitated as above. Unlabeled IL-5 and chemokines were purchased from R&D Systems, Minneapolis, MN. All measurements were determined in triplicate and repeated in at least two independent experiments. The K_d values were determined using the GraphPad Prism data analysis software package (GraphPad software, San Diego, CA).

Neutrophil preparation and intracellular calcium measurements. Granulocytes were prepared from whole peripheral blood of healthy donors by Ficoll-Hypaque discontinuous gradient centrifugation and dextran sedimentation followed by hypotonic lysis of the remaining red blood cells. Neutrophils, at a concentration of 1.5 x 10^6 cells/ml, were loaded with 2.5 μM Fura II-AM (Molecular Probes, Eugene, OR) in PBS for 45 min at 37°C. Cells were washed twice with PBS and suspended in PBS at a final concentration of 1.5 x 10^6 cells/ml. One ml of neutrophils was mixed with 1 ml HBSS for further analysis of calcium flux responses. The cuvette containing 2 ml of cells was continuously stirred at 37°C in a Model MS-III ratio fluorescence spectrophotometer (Photon Technology International, Inc., London, Ontario). The cells were stimulated with IL-8 (Peprotech, Rocky Hill, NJ), N-formyl-met-leu-phe (fMLF) (Sigma Chemical Co., St. Louis, MO), and/or supernatants from insect cells expressing M3 protein or LacZ protein. The calcium flux response was measured continuously every 200 msec as a relative fluorescence ration of excitation at 340 nm and 380 nm with an emission at 510 nm.

M3 protein binds chemokines. To detect chemokine binding proteins in the supernatant from γHV68 infected cells, supernatants were collected 24 hours after γHV68 or mock infections, mixed with various chemokines and chemically cross linked. Binding of proteins to the M3 protein was detected after analysis by denaturing gel electrophoresis and western blotting with anti-M3 polyclonal antisera. A supershifted M3 protein containing complex was observed when RANTES, MCP-1, or IL8 were added to supernatants from γHV68 infected cells. Similar complexes were not seen

when mock infected supernatants were used. Incubated with 100pM ^{125}I-RANTES or ^{125}I-interleukin-5 (IL-5), and immunoprecipitated with polyclonal rabbit sera raised against bacterially expressed M3 protein. ^{125}I-RANTES was precipitated by M3 protein specific antiserum, but not pre-immune antibody, from infected cell supernatants, but not from mock infected supernatants. IL-5 was not precipitated from the γHV68 supernatants by anti-M3 antibody, demonstrating specificity of M3 protein binding to RANTES. In addition, supernatants harvested from Sf9 cells infected with either an M3 protein or β-galactosidase expressing baculoviruses were assayed. Anti-M3 protein antibody precipitated ^{125}I RANTES from supernatants from cells infected with the M3 protein expressing baculovirus, but not the β-galactosidase expressing baculovirus. Together these experiments demonstrate that the γHV68 M3 protein binds RANTES in the absence of other γHV68 encoded proteins.

M3 protein binds CC chemokines with high affinity. To measure the affinity of the interaction between the M3 protein and RANTES, a saturation binding experiment was performed, keeping the concentration of M3 protein constant and altering the concentration of ^{125}I-RANTES. Conditions of the assay were selected such that input M3 protein was quantitatively precipitated. The interaction of ^{125}I-RANTES and M3 protein was saturable and of high affinity (K_d=1.6±0.12 nM,). Competition for binding of ^{125}I-RANTES to M3 protein was used to determine if the M3 protein binds other chemokines, and to determine the binding affinity of unlabeled RANTES, as well as several other CC and CXC chemokines. Preincubation of ^{125}I-RANTES with increasing amounts 1a led to dose dependent inhibition of binding of ^{125}I-RANTES to M3 protein, while increasing amounts of unlabeled IL-5 had no effect. Interestingly, while the M3 protein binds the human CXC chemokine IL-8, six other murine CXC chemokines failed to efficiently compete for bind of ^{125}I-RANTES to the M3 protein (Kd.1.0 μM for all six). These data show that the M3 protein binds human IL-8 as well as several human and mouse CC chemokines with high affinity, but fails to interact with multiple mouse CXC chemokines.

The M3 protein binds multiple CC chemokines, fractalkine, and lymphotactin, but not murine CXC chemokines, with high affinity. As described in van Berkel, *et al.*, J. Virol. 2000 74(15): 6741-6747 (incorporated herein by reference), competition for binding of ^{125}I-hRANTES to M3 protein was used to determine if the M3 protein binds other chemokines and to determine the binding affinity of unlabeled hRANTES, as well

as several murine CC and CXC chemokines, Preincubation of ^{125}I-hRANTES with increasing amounts of the unlabeled CC chemokines hRANTES, mMCP-1, and mMIP - 1 led to dose-dependent inhibition of binding of ^{125}I-hRANTES to M3 protein (Fig. 5A), while increasing amounts of unlabeled IL-5 had no effect (data not shown). Similar competition experiments with m-fractalkine and m-lymphotactine demonstrated that both of these chemokines bound with high affinity (Fig. 5B). Interestingly, while the M3 protein bound the human CXC chemokine IL-8 (Fig. 5C), seven murine CXC chemokines failed to efficiently compete for binding of ^{125}I-hRANTES to the M3 protein (K_d.>1.0 μM for all seven, Fig. 5C). These data show that the M3 protein binds with high affinity to (i) the CC chemokines hRANTES, mMIP-1 , and mMCP-1, (ii) the murine C and CX_3C chemokines lymphotacting and fractalkine, and (iii) the CXC chemokine hIL-8, but not the test murine CXC chemokines. The binding of fractalkine by M3 polypeptide indicates that M3 polypeptides of the invention will be active in binding and blocking the signaling of fractalkine in various central nervous system infections and diseases.

M3 protein blocks chemokine mediated calcium flux. To test the functional consequence of M3 protein chemokine binding, calcium flux in human neutrophils stimulated with IL-8 and the non-chemokine chemoattractant fMLF, which activate distinct G protein-coupled receptors was monitored. Both receptors are coupled to calcium mobilization which can be monitored in real time in Furo-II loaded cells. Both IL-8 and fMLF induced a robust calcium flux (EC_{50} ~1 nm, consistent with previous reports. In contrast, supernatants from insect cells expressing M3 protein or LacZ did not induce calcium flux when tested at a final concentration of M3 protein of 5 nM or an equivalent volume of the LacZ supernatant. Thus the M3 protein did not act as a calcium-mobilixing agonist of neutrophils. Instead the M3 protein, when added to the cells after IL-8, was able to specifically block the normal IL-8 induced calcium flux by ~90%, whereas it did not affect signaling by fMLF. Further, IL-8 signaling was completely blocked by premixing the M3 protein with IL-8 for two minutes prior to addition to the cells. In additional experiments not shown, RANTES induction of calcium flux via activation of the RANTES receptors CCR5 expressed in human embryonic kidney (HEK) 293 cells was completely abolished by pretreatment of the cells or premixing of RANTES with M3 protein, whereas little to no effect was observed

on calcium signaling by STP through an endogenous nucleotide receptor. These results indicate that the M3 protein is a potent inhibitor of IL-8 and RANTES action.

Restenosis. M3 polypeptide was tested for efficacy in reducing atherosclerotic plaque deposition in a model of coronary restenosis after balloon angioplasty, as described in Liu *et al.*, S. Clin. Invest. **105** 1613-1621 (2000). Atherosclerotic plaque formation is critically involved in vascular occlusion and has been linked to excessive inflammatory and thrombotic response to arterial injury. The results, as shown in Tables I-III and Fig. 4, demonstrate that M3 polypeptide is effective in reducing atherosclerotic plaque *in vivo*.

TABLE I. Statistical Analysis of Plaque Area

	DF	Sum of Square	Mean Square	F-Value	P-Value
AVOVA	3	.015	.005	1.632	.1909
Residual	63	.195	.003		

Model II estimate of between component variance: 1.67E-4
5 cases were omitted due to missing values.

TABLE II. AVOVA Effect; Means for Plaque Area

Infusion	Count	Mean	Std. Dev.	Std. Err.
Saline	16	.052	.079	.020
M3 3pg	16	.037	.054	.013
M3 3ng	17	.021	.048	.012
M3 300ng	18	.013	.034	.008

5 cases were omitted due to missing values.

TABLE III. AVOVA Effect; Fisher's PLSD for Plaque area; Significance Level: 5%

Infusion	Mean Diff.	Crit. Diff	P-Value
Saline, M3 3pg	.015	.039	.4589
Saline, M3 3ng	.031	.039	.1144
Saline, M3 300ng	.039	.038	.0459
M3 3pg, M3 3ng	.016	.039	.4016
M3 3pg, M3 300ng	.024	.038	.2087
M3 3ng, M3 300ng	.008	.038	.6754

5 cases were omitted due to missing values.

Cloning Additional M3 Genes. Based on the amino acid sequences provided by the present invention degenerate oligonucleotide primers containing restriction sites will be synthesized. First strand cDNA will be synthesized from RNA prepared from a tissue of interest and PCR will be performed with an initial five cycles, for example, at 37 for 60s, followed by 25 cycles at 50 for 60s (denaturation at 95 for 30s and extension at 72

for 90s) in order to amplify a M3 cDNA fragment that can be subsequently subcloned into Bluescript II KS (Stratagene). Construction of a cDNA library using poly A RNA isolated from the selected tissue will be performed using Stratagene ZAP Express Vector according to the directions of the manufacturer. Potential clones will be subsequently amplified and an aliquot of this cDNA library containing phage will be screened with the M3 cDNA that has been 32P- labeled with Klenow enzyme. Isolated phagemids will be subsequently subjected to automated sequencing on both strands using Applied Biosystems Instrumentation (model 373a) and the dye- terminator protocol. Sequence analysis will be performed using software developed by the University of Wisconsin genetics computer group (Altschul, *et al. J. Mol. Biol.* 215:403-410, 1990).

DNA and RNA analysis. RNA will be isolated by CsCl centrifugation in guanidine isothiocyanate (Chirgwin, *et al.* (1979) Biochemistry. 18:5294-9). Biologically active ribonucleic acid will be isolated from sources enriched in ribonuclease. DNA will be isolated from these gradients as well. In some cases, RNA will be isolated using RNAzol (Biotecx Lab, Inc.) according to the directions of the manufacturer. Poly A RNA will be enriched by elution through an oligo dT column (Pharmacia). For example, 10 mcg of total RNA, 2 mcg of poly A RNA, or 10 mcg of restriction endonuclease cut DNA will be electrophoresed in agarose, and transferred to Gene Screen (NEN Dupont) membranes. Membranes will be hybridized with ^{32}P labeled full length cDNA or a fragment encoding the translated protein. High stringency hybridization will be performed, for example, in 50% formamide, 10% dextran sulfate, 5X SSC, 1X Denhardt's solution (0.0002% (w/v) polyvinylpyrrolidone, 0.0002% (w/v) BSA, 0.0002% (w/v) Ficoll 400), 1% (w/v) SDS, 100 mcg/ml denatured herring sperm DNA, and 20mM Tris at 42oC and blots will be washed with 0.2X SSC, 0.5% SDS at 65oC. Low stringency hybridization will be performed, for example, in 0.6M NaCl, 80mM TrisCl, 4mM EDTA, 0.1% (w/v) sodium pyrophosphate, 0.1% (w/v) SDS, 10X Denhardts, 100mcg/ml denatured herring sperm DNA at 50oC and washed with 1XSSC, 0.05% SDS at 50oC. Quantitation of the intensity of band hybridization will be determined using a Phosphor-Imager (Molecular Dynamics).

M3 *Gene Analysis.* A cDNA probe from the coding region of the M3 cDNA will be ^{32}P-labeled with Klenow enzyme and used to screen approximately 1 X 106 plaques from a mammalian genomic library (*e.g.*, a variety of libraries are available from Stratagene, La Jolla, CA) under conditions of low stringency (hybridization in 0.6 M NaCl, 80mM TrisCl, 4mM EDTA, 0.1% sodium pyrophosphate, 0.1% SDS, 10X DenhardtOs solution (0.002% polyvinylpyrrolidone, 0.002% BSA, 0.002% Ficoll 400), 100mcg/ml denatured herring sperm DNA at 50 C and blots washed with 1X SSC, 0.05% SDS at 50oC). Plaques that hybridize strongly will be purified. The genomic DNA will be liberated from the phage DNA by restriction digestion with the appropriate enzymes and sub-cloned into pBlue- Script SK II (Stratagene). Any positively identified genomic fragment that hybridizes with the probe will be subcloned into, for example, pBlue-Script KS II, and subjected to automated sequencing on both strands using Applied Biosystems Instrumentation (model 373a) and the dye-terminator protocol. Sequence analysis will be performed using software developed by the University of Wisconsin genetics computer group Altschul, *et al.* (1990) J. Mol. Biol. 215, 403-410.

The M3 gene homolog chromosomal localization will be determined by the analysis of any identified polymorphisms in the gene. PCR primers flanking this polymorphism will be constructed and genomic DNA will be amplified by PCR. Using these primers, a size polymorphism will be identified (For example, see Rowe, *et al.*, *Mamm. Gen.* 5, 253-274, 1994).

Murine M3 cDNA Analysis. The identified genomic fragment will be used to screen a mammalian cDNA expression library (Stratagene) under conditions of high stringency (hybridization in 50% formamide, 10% dextran sulfate, 5X SSC, 1X DenhardtOs solution, 1% SDS, 100 mcg/ml denatured herring sperm DNA, and 20mM Tris at 42 C and blots were washed with 0.2X SSC, 0.5% SDS at 65 C). Positive plaques will be identified, purified, and phagemids will be prepared according to the instructions of the library manufacturer. The inserts will be completely sequenced on both strands by automated sequencing. Alignment analysis will be determined by the Clustal method using MegAlign software (DNASTAR Inc) (Higgins, *et al.* (1988) Gene 73, 237-244).

Construction and Transfection of Protein Expression Vectors. PCR primers will be designed to amplify the coding region of the M3 gene, or homolog or derivative thereof, flanked by convenient restriction sites for subsequent sub-cloning. PCR will be performed under standard conditions using M3 cDNA-pBlueScript as a template. The resulting PCR products will be subsequently subcloned using, for example, a TA cloning kit (Invitrogen, San Diego, CA) and confirmatory sequencing will be performed. M3 cDNA will be subcloned into the available restriction sites of pcDNA-I/Amp (Invitrogen). Approximately 4 mcg of the M3-pcDNA-I construct will be transfected into 100 mm plates containing ~30% confluent COS cells using DEAE-Dextran (Lopata, *et al., Nucl. Acids Res.* 12, 5707-5717, 1984). In order to measure transfection efficiency, a replicate sample of COS cells will be transfected with a CMV promoter-placental alkaline phosphatase control plasmid (Fields-Berry, *et al., Proc. Nati. Acad. Sci. U.S.A.* 89, 693-697, 1992). RNA expression will be confirmed by Northern analysis using the M3 cDNA as a probe. M3-pcDNA-I transfected or mock transfected COS cell supernatant will be obtained after 72 hrs of culture and stored at 4 C. In another set of transfection experiments, M3 cDNA will be similarly subcloned into the available site of MoLTR-SV40 I/PA expression vector (for example, see Luster, *et al., J. Exp. Med.* 178, 1057-1065, 1993). 20 mcg of linearized M3-MoLTR construct and 1 mcg of linearized neomycin resistance plasmid pSV7Neo will be used to transfect J558L cells by electroporation. G418 resistant cells from single wells will be analyzed for M3 mRNA expression by Northern analysis. Cells expressing M3 virus protein or control untransfected cells (that do not express M3 protein) will be expanded in large cultures. In order to optimize the concentration of M3 virus protein in the supernatant, the cells will be grown at high density (1 X 106 cells/ml) in RPMI without FCS, cultured for 72 hrs, and the conditioned medium will be concentrated 5-fold with Centricon 3000 microconcentrators (Amicon, Beverly, MA) before being stored.

Chemotaxis Assays. Murine leukocytes, for example eosinophils, will be isolated. Eosinophils in particular will be isolated from IL-5 transgenic mice (Dent, *et al.* (1990) *J. Exp. Med.* 172, 1425-1431). These mice develop splenomegaly with eosinophils accounting for ~30% of the splenocytes. Eosinophils will be purified from the spleen using immuno-magnetic separation to remove the contaminating splenocytes. Briefly, splenocytes will be labeled with anti-Thy-1 (M5/49), anti-B220 (6B2), and

anti-Lyt-2 (53-6.7). Hybridoma cell lines will be obtained, for example, from American Type Culture Collection and hybridoma cell supernatants will be used as a source of antibodies. The antibody labeled cells will be treated with, anti- serum coated-magnetic beads, the anti-serum having specificity for the isotype of the primary antibody, (M450, Dynal, Great Neck, NY) and eosinophils will be enriched by negative selection through a magnetic field. Macrophage cells will be isolated from the peritoneal cavity of mice that had been pre-treated (2 days prior) with intraperitoneal injection of 2.9% thioglycollate (Difco, Detroit, CA). Peritoneal neutrophils will be isolated from mice pre-treated with sodium casein (Luo, *et al.*, *J. Immun.* 153, 4616-4624, 1994). Macrophages and neutrophils will be purified by Percoll gradients (Luo, *et al.*, *J. Immun.* 153, 4616-4624, 1994. Eosinophils or macrophages will be suspended in HBSS with 0.05% BSA at 2 X 106 cells/ml, respectively, and 50 ml of replicate cells will be placed in the top well of a 48 well micro-chemotaxis chamber (Neuro Probe, Inc, Cabin John, MD). A polycarbonate filter with 5-μm pores will be used to separate the cells from buffer (30 ml) alone or buffer containing recombinant COS cell supernatant and a positive (for example MCP-1 (Rollins, & Pober, *Am. J. Path.*, 138, 1315-1319, 1991)) and negative control (for example, supernatant from mock-transfected COS cells) so that a comparison can be made and the immunosuppressive (inhibition of chemotaxis) properties of the M3 protein assessed. Cells will be incubated at 37oC for 60 minutes (eosinophils and neutrophils) or 90 minutes (macrophages) and the cells that migrate across the filter and adhere to the bottom side of the filter will be stained with Diff-Quick (Baxter Scientific, McGaw Park, IL). The number of cells per 400X field will be counted.

Statistical Analysis. The statistical significance of differences between means will be determined by analysis of variance (ANOVA). $P < 0.05$ will be considered significant. When ANOVA indicates a significant difference, the Newman-Keuls test will be used to determine which groups are significantly different from each other.

Analysis of M3 cDNA. Using degenerate oligonucleotide primers based upon the amino acid sequence of the M3 gene sequences disclosed herein, a cDNA will be amplified by PCR from single stranded cDNA from a selected mammalian tissue. This PCR product will encode a peptide identical to the M3 gene and will be used to screen

an amplified cDNA library made from selected mammalian tissue. Positive plaques will be subsequently purified. Sequence analysis of the plaques will then be preformed as described herein above.

M3 *mRNA Expression in Different Organs*. Northern blot analysis of total RNA isolated from different mammalian tissue samples may reveal detectable expression of any M3 virus gene, homolog or derivative. Mammalian tissues including the brain, bone marrow, skin, intestines, stomach, heart, thymus, lymph node, mammary gland, skeletal muscle, tongue, spleen, liver, testes, and kidney will be analyzed. Likewise, cell lines such as macrophages isolated and cultured from the spleen, a lung epithelial cell line, and a colon adenocarcinoma cell, will be analyzed for expression of M3 or homologous mRNA.

Other Embodiments

In other preferred embodiments, the invention includes any protein which is substantially identical to a M3 virus polypeptide (preferably having the sequence of Figure 3); such homologs include other substantially pure naturally occurring mammalian homologs of M3 proteins as well as allelic variations; natural mutants; induced mutants; proteins encoded by DNA that hybridizes to the M3 sequences of Figures 1 or 2 under high stringency conditions or low stringency conditions (*e.g.*, washing at 2X SSC at 40°C with a probe of at least 40 nucleotides); and polypeptides or proteins specifically bound by antisera directed to a M3 polypeptide. The term also includes chimeric polypeptides that include a M3 fragment.

The invention further includes analogs of the M3 polypeptides. Analogs can differ from the naturally occurring M3 protein by amino acid sequence differences, by post-translational modifications, or by both. Analogs of the invention will generally exhibit at least 70%, more preferably 80%, even more preferably 90%, and most preferably 95% or even 99%, identity with all or part of a naturally occurring M3 protein sequence. The length of comparison sequences will be at least 8 amino acid residues, preferably at least 24 amino acid residues, and more preferably more than 35 amino acid residues. Modifications include *in vivo* and *in vitro* chemical derivatization of polypeptides, *e.g.*, acetylation, carboxylation, phosphorylation, or glycosylation; such modifications may occur during polypeptide synthesis or processing or following

treatment with isolated modifying enzymes. Analogs can also differ from the naturally occurring M3 polypeptide by alterations in primary sequence. These include genetic variants, both natural and induced (for example, resulting from random mutagenesis by irradiation or exposure to ethanemethylsulfate or by site-specific mutagenesis as described in Sambrook, Fritsch and Maniatis, *Molecular Cloning: A Laboratory Manual* (2d ed.), CSH Press, 1989, hereby incorporated by reference; or Ausubel *et al.*, *supra*, hereby incorporated by reference). Also included are cyclized peptides molecules and analogs which contain residues other than L-amino acids, *e.g.*, D-amino acids or non-naturally occurring or synthetic amino acids, *e.g.*, or amino acids.

In addition to full-length polypeptides, the invention also includes M3 polypeptide fragments. As used herein, the term "fragment" means at least 10 contiguous amino acids, preferably at least 30 contiguous amino acids, more preferably at least 50 contiguous amino acids, and most preferably at least 60 to 80 or more contiguous amino acids. Fragments of M3 proteins can be generated by methods known to those skilled in the art or may result from normal protein processing (*e.g.*, removal of amino acids from the nascent polypeptide that are not required for biological activity or removal of amino acids by alternative mRNA splicing or alternative protein processing events).

Preferable fragments or analogs according to the invention are those which exhibit biological activity (for example, the ability to act as an immunomodulator as described herein). Preferably, a M3 polypeptide, fragment, or analog exhibits at least 10%, more preferably 30%, and most preferably, 70% or more of the biological activity of a full length naturally occurring M3 polypeptide.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure come within known or customary practice within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth.

CLAIMS

1. A method of immunomodulation in a mammal, said immunomodulation method comprising administering to said mammal a therapeutically effective amount of an M3 polypeptide, wherein said polypeptide has an immunomodulatory effect in said mammal.

2. The method of claim 1, wherein the M3 polypeptide comprises an amino acid sequence substantially identical to SEQ ID NO:3.

3. The method of claim 1, wherein the M3 polypeptide comprises an amino acid sequence having about 80% amino acid sequence identity to SEQ ID NO:3.

4. The method of claim 1, wherein said immunomodulation is immunosuppression, immunostimulation, cell proliferation, apoptosis, decreasing T cell stimulation, or decreasing inflammation in a mammal.

5. The method of claim 1, wherein said mammal is a human.

6. The method of claim 1, wherein said M3 polypeptide is a multi-transmembrane receptor related protein.

7. The method of claim 1, wherein said mammal has a condition selected from acute inflammation, rheumatoid arthritis, transplant rejection, restenosis, asthma, allergies, inflammatory bowel disease, uveitis, psoriasis, atopic dermatitis, bronchial asthma, pollinosis, systemic lupus erythematosus, nephrotic syndrome lupus, multiple sclerosis, myasthenia gravis, type I and type II diabetes mellitus, glomerulonephritis, Hashimoto's thyroiditis, autoimmune hemolytic anemia, autoimmune thrombocytopenic purpura, microbial infection, malignancy and metastasis, autoimmune disease, cirrhosis, endotoxemia, atherosclerosis, reperfusion injury and inflammatory responses, AIDS, cirrhosis of the liver, neurodegeneration, myelodysplastic syndrome, ischemic injury, central nervous system infection, or central nervous system disease.

8. The method of claim 1, wherein the method further comprises providing an M3 nucleic acid molecule under the control of a promoter providing controllable expression of said M3 nucleic acid molecule, wherein said M3 nucleic acid molecule is expressed in a construct capable of delivering a therapeutically effective amount of M3 polypeptide.

9. The method of claim 8, wherein said M3 nucleic acid molecule comprises a nucleic acid sequence of SEQ ID NO:1 or SEQ ID NO:2.

10. The method of claim 8, wherein said M3 nucleic acid molecule comprises a nucleic acid sequence that is substantially identical to SEQ ID NO:1.

11. The method of claim 8, wherein said M3 nucleic acid molecule comprises a nucleic acid sequence that is substantially identical to SEQ ID NO:2.

12. The method of claim 9, wherein said M3 nucleic acid molecule comprises a nucleotide having about 80% nucleotide sequence identity to SEQ ID NO:1.

13. The method of claim 9, wherein said M3 nucleic acid molecule comprises a nucleotide having about 80% nucleotide sequence identity to SEQ ID NO:2.

14. A method of reducing inflammation in a mammal, said method comprising administering a therapeutically effective amount of an anti-M3 polypeptide neutralizing antibody to a mammal.

15. A method of modulating chemokine receptor function, said method comprising contacting an M3 polypeptide with a mammalian chemokine such that chemokine receptor function is modulated.

16. The method of claim 15, wherein the M3 polypeptide comprises an amino acid sequence substantially identical to SEQ ID NO:3.

17. The method of claim 15, wherein the M3 polypeptide comprises an amino acid sequence having about 80% amino acid sequence identity to SEQ ID NO:3.

18. The method of claim 15, wherein the mammalian chemokine receptor is a human chemokine receptor.

19. The method of claim 15, wherein said M3 polypeptide is a multi-transmembrane receptor related protein.

20. The method of claim 15, wherein the method further comprises providing a cell that expresses a M3 nucleic acid molecule, wherein said M3 nucleic acid molecule is expressed in a construct capable of delivering a therapeutically effective amount of M3 polypeptide.

21. The method of claim 20, wherein said M3 nucleic acid molecule comprises a nucleic acid sequence of SEQ ID NO:1 or SEQ ID NO:2.

22. The method of claim 20, wherein said M3 nucleic acid molecule comprises a nucleic acid sequence that is substantially identical to SEQ ID NO:1.

23. The method of claim 20, wherein said nucleic acid molecule comprises a nucleic acid sequence that is substantially identical to SEQ ID NO:2.

24. The method of claim 21, wherein said nucleic acid molecule comprises a nucleotide having about 80% nucleotide sequence identity to SEQ ID NO:1.

25. The method of claim 21, wherein said nucleic acid molecule comprises a nucleotide having about 80% nucleotide sequence identity to SEQ ID NO:2.

26. A method of inhibiting intimal hyperplasia in a mammal, said method comprising administering a therapeutically effective amount of an M3 polypeptide to a mammal having intimal hyperplasia such that the intimal hyperplasia is inhibited.

27. The method of claim 26, wherein the M3 polypeptide comprises an amino acid sequence substantially identical to SEQ ID NO:3.

28. The method of claim 26, wherein the M3 polypeptide comprises an amino acid sequence having about 80% amino acid sequence identity to SEQ ID NO:3.

29. The method of claim 26, wherein the mammal is a human.

30. The method of claim 26, wherein the mammal has a vascular injury.

31. The method of claim 26, wherein the method further comprises providing an M3 nucleic acid molecule under the control of a promoter providing controllable expression of said M3 nucleic acid molecule, wherein said M3 nucleic acid molecule is expressed in a construct capable of delivering a therapeutically effective amount of M3 polypeptide.

32. The method of claim 31, wherein said nucleic acid molecule comprises a nucleic acid sequence of SEQ ID NO:1 or SEQ ID NO:2.

33. The method of claim 31, wherein said nucleic acid molecule comprises a nucleic acid sequence that is substantially identical to SEQ ID NO:1.

34. The method of claim 31, wherein said nucleic acid molecule comprises a nucleic acid sequence that is substantially identical to SEQ ID NO:2.

35. The method of claim 32, wherein said nucleic acid molecule comprises a nucleotide having about 80% nucleotide sequence identity to SEQ ID NO:1.

36. The method of claim 32, wherein said nucleic acid molecule comprises a nucleotide having about 80% nucleotide sequence identity to SEQ ID NO:2.

37. A method of binding chemokines, said method comprising contacting a chemokine with a M3 polypeptide.

38. The method of claim 37, wherein the chemokine is a mammalian chemokine.

39. The method of claim 38, wherein the chemokine is a human chemokine.

40. A pharmaceutical composition comprising a therapeutically effective amount of an M3 polypeptide.

41. The pharmaceutical composition of claim 40, wherein the M3 polypeptide comprises an amino acid sequence substantially identical to SEQ ID NO:3.

42. The pharmaceutical composition of claim 40, wherein the M3 polypeptide comprises an amino acid sequence having about 80% amino acid sequence identity to SEQ ID NO:3.

43. A pharmaceutical composition comprising a therapeutically effective amount of an M3 nucleic acid molecule.

44. The pharmaceutical composition of claim 43, wherein said M3 nucleic acid molecule comprises a nucleic acid sequence of SEQ ID NO:1 or SEQ ID NO:2.

45. The pharmaceutical composition of claim 43, wherein said M3 nucleic acid molecule comprises a nucleic acid sequence that is substantially identical to SEQ ID NO:1.

46. The pharmaceutical composition of claim 43, wherein said M3 nucleic acid molecule comprises a nucleic acid sequence that is substantially identical to SEQ ID NO:2.

47. The pharmaceutical composition of claim 44, wherein said M3 nucleic acid molecule comprises a nucleotide having about 80% nucleotide sequence identity to SEQ ID NO:1.

48. The pharmaceutical composition of claim 44, wherein said M3 nucleic acid molecule comprises a nucleotide having about 80% nucleotide sequence identity to SEQ ID NO:2.

49. A diagnostic kit for detecting the presence of an M3 polypeptide in a biological sample comprising, an M3 primer or an antibody that specifically binds an M3 polypeptide, ancillary reagents suitable for use in detecting the presence of M3 polypeptide, and instructions for using the kit.

SEQ ID NO:1

CTGGGAGAGCGTCAGCCATGGCCTTCCTATCCACATCTGTGCTCATTAAATGC
TGCATCCTCCTGTTGGCAGGAGGATTGGCTGAGAGCCTTACTCTAGGTTTGGC
ACCTGCTCTGTCTACCCACTCTTCTGGAGTGTCAACACAGTCTGTTGATTTGTC
ACAAATTAAAAGAGGCGATGAGATCCAAGCCCACTGTCTGACACCAGCAG
AAACTGAAGTGACTGAGTGTGCTGGCATTCTGAAAGATGTTCTGTCAAAAAA
CCTTCATGAGCTTCAAGGGCTCTGTAATGTTAAGAACAAAATGGGTGTACCA
TGGGTTTCTGTGGAAGAGCTGGGTCAAGAGATTATAACAGGCAGATTGCCAT
TCCCCTCTGTGGGTGGCACACCAGTGAATGACCTGGTACGAGTTTTGGTGGTG
GCAGAATCAAACACCCCAGAAGAGACTCCAGAGGAGGAGTTTTATGCCTATG
TGGAACTTCAGACTGAGTTGTACACCTTTGGTCTGTCTGATGACAATGTGGTT
TTCACTAGCGATTATATGACTGTGTGGATGATTGACATTCCCAAATCTTATGT
GGATGTGGGCATGCTCACACGAGCAACTTTCCTGGAGCAGTGGCCGGGTGCC
AAAGTGACTGTCATGATTCCCTACTCCTCCACCTTTACCTGGTGTGGAGAGCT
TGGCGCCATCTCTGAAGAATCTGCCCCACAACCAAGTTTGAGTGCCAGATCC
CCGGTCTGTAAGAACAGTGCCAGATATTCTACCTCAAAATTTTGCGAGGTAG
ATGGATGCACAGCTGAAACTGGCATGGAAAAGATGTCTCTGCTCACTCCATT
TGGAGGCCCACCTCAACAGGCTAAGATGAACACTTGCCCATGCTACTACAAG
TACAGCGTGAGCCCACTGCCTGCCATGGACCACTTGATCCTAGCTGACCTAG
CTGGCCTGGATTCTCTCACTTCACCTGTTTATGTGATGGCAGCATATTTTGATT
CTACACATGAAAATCCTGTGAGACCATCCAGCAAGCTTTATCATTGTGCTCTA
CAGATGACAAGTCATGATGGGGTGTGGACTTCAACTTCCAGTGAGCAGTGTC
CCATTAGACTGGTTGAGGGCCAGTCTCAAAATGTGCTGCAGGTCCGGGTGGC
TCCGACATCGATGCCTAACCTGGTTGGGGTCAGCTTGATGTTGGAGGGGCAA
CAGTACAGGCTGGAGTATTTTGGGGATCATTGATACTCAGTAACTTAAGAGG
AAATTTTTTTGTTAAAAATAAAGTTATTTTT

Fig. 1

SEQ ID NO:2

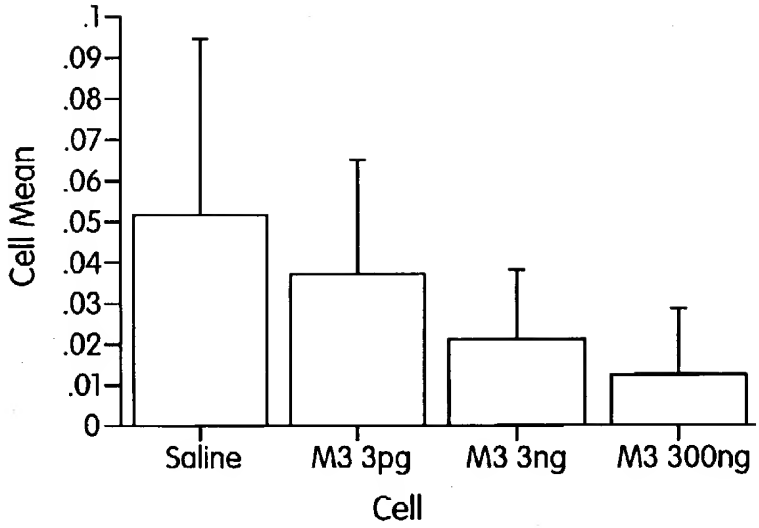
ATGGCCTTCCTATCCACATCTGTGCTCATTAAATGCTGCATCCTCCTGTTGGC
AGGAGGATTGGCTGAGAGCCTTACTCTAGGTTTGGCACCTGCTCTGTCTACCC
ACTCTTCTGGAGTGTCAACACAGTCTGTTGATTTGTCACAAATTAAAAGAGGC
GATGAGATCCAAGCCCACTGTCTGACACCAGCAGAAACTGAAGTGACTGAGT
GTGCTGGCATTCTGAAAGATGTTCTGTCAAAAAACCTTCATGAGCTTCAAGG
GCTCTGTAATGTTAAGAACAAAATGGGTGTACCATGGGTTTCTGTGGAAGAG
CTGGGTCAAGAGATTATAACAGGCAGATTGCCATTCCCCTCTGTGGGTGGCA
CACCAGTGAATGACCTGGTACGAGTTTTGGTGGTGGCAGAATCAAACACCCC
AGAAGAGACTCCAGAGGAGGAGTTTTATGCCTATGTGGAACTTCAGACTGAG
TTGTACACCTTTGGTCTGTCTGATGACAATGTGGTTTTCACTAGCGATTATAT
GACTGTGTGGATGATTGACATTCCCAAATCTTATGTGGATGTGGGCATGCTCA
CACGAGCAACTTTCCTGGAGCAGTGGCCGGGTGCCAAAGTGACTGTCATGAT
TCCCTACTCCTCCACCTTTACCTGGTGTGGAGAGCTTGGCGCCATCTCTGAAG
AATCTGCCCCACAACCAAGTTTGAGTGCCAGATCCCCGGTCTGTAAGAACAG
TGCCAGATATTCTACCTCAAAATTTTGCGAGGTAGATGGATGCACAGCTGAA
ACTGGCATGGAAAAGATGTCTCTGCTCACTCCATTTGGAGGCCCACCTCAAC
AGGCTAAGATGAACACTTGCCCATGCTACTACAAGTACAGCGTGAGCCCACT
GCCTGCCATGGACCACTTGATCCTAGCTGACCTAGCTGGCCTGGATTCTCTCA
CTTCACCTGTTTATGTGATGGCAGCATATTTTGATTCTACACATGAAAATCCT
GTGAGACCATCCAGCAAGCTTTATCATTGTGCTCTACAGATGACAAGTCATG
ATGGGGTGTGGACTTCAACTTCCAGTGAGCAGTGTCCCATTAGACTGGTTGA
GGGCCAGTCTCAAAATGTGCTGCAGGTCCGGGTGGCTCCGACATCGATGCCT
AACCTGGTTGGGGTCAGCTTGATGTTGGAGGGGCAACAGTACAGGCTGGAGT
ATTTTGGGGATCAT

Fig. 2

SEQ ID NO: 3

MAFLSTSVLIKCCILLLAGGLAESLTLGLAPALSTHSSGVSTQSVDLSQIKRGDEIQ
AHCLTPAETEVTECAGILKDVLSKNLHELQGLCNVKNKMGVPWVSVEELGQEII
TGRLPFPSVGGTPVNDLVRVLVVAESNTPEETPEEEFYAYVELQTELYTFGLSDD
NVVFTSDYMTVWMIDIPKSYVDVGMLTRATFLEQWPGAKVTVMIPYSSTFTWC
GELGAISEESAPQPSLSARSPVCKNSARYSTSKFCEVDGCTAETGMEKMSLLTPF
GGPPQQAKMNTCPCYYKYSVSPLPAMDHLILADLAGLDSLTSPVYVMAAYFDS
THENPVRPSSKLYHCALQMTSHDGVWTSTSSEQCPIRLVEGQSQNVLQVRVAPT
SMPNLVGVSLMLEGQQYRLEYFGDH

Fig. 3



5 cases were omitted due to missing values.

Fig. 4

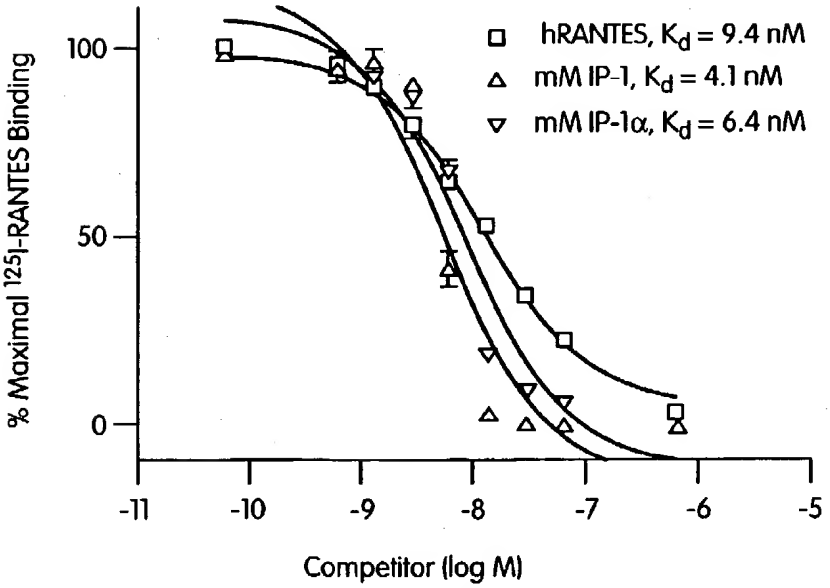


Fig. 5A

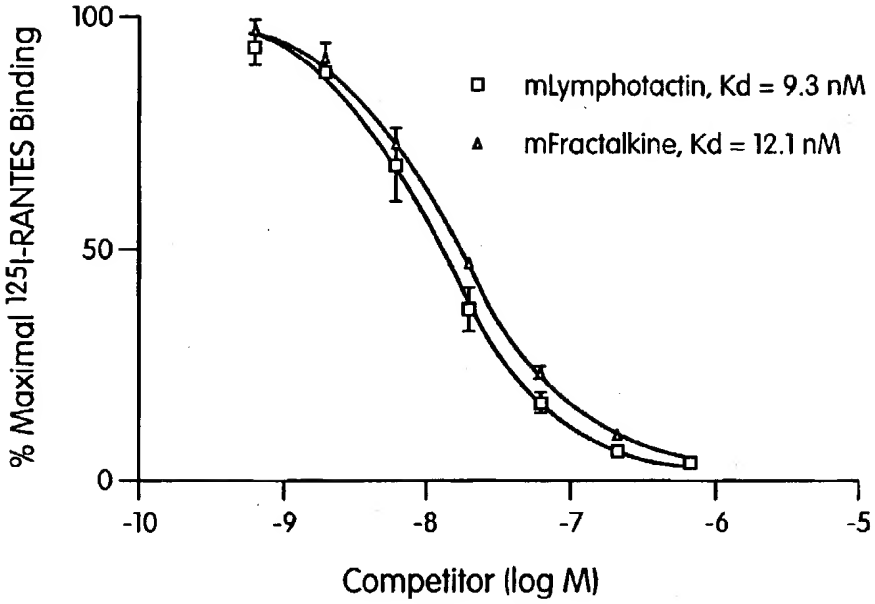


Fig. 5B

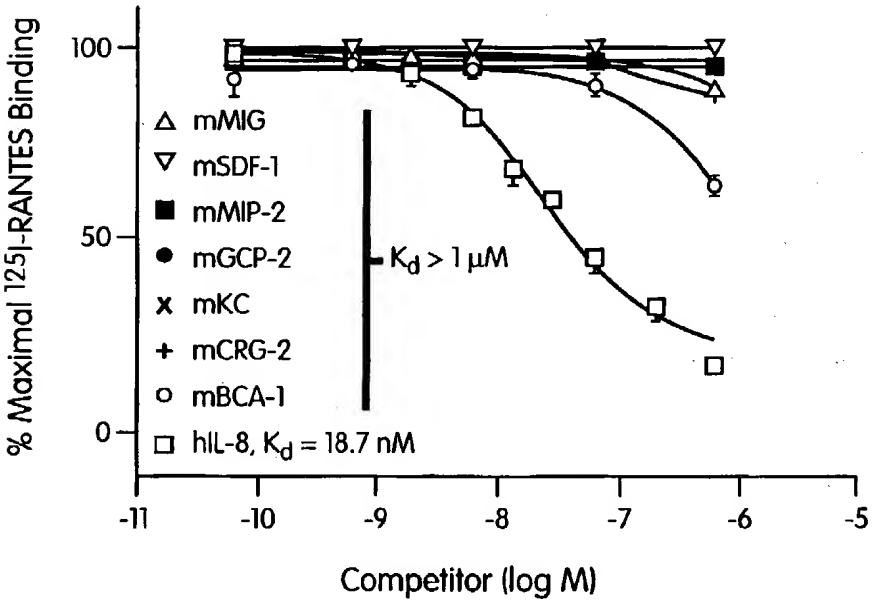


Fig. 5C

SEQUENCE LISTING

<110> John P. Robarts Research Institute
Washington State University

<120> THERAPEUTIC USES OF M3 POLYPEPTIDE

<130> 50082/014WO2

<150> US 60/154,799
<151> 1999-09-20

<160> 3

<170> FastSEQ for Windows Version 4.0

<210> 1
<211> 1287
<212> DNA
<213> Gamma herpesvirus

<400> 1
ctgggagagc gtcagccatg gccttcctat ccacatctgt gctcattaaa tgctgcatcc 60
tcctgttggc aggaggattg gctgagagcc ttactctagg tttggcacct gctctgtcta 120
cccactcttc tggagtgtca acacagtctg ttgatttgtc acaaattaaa agaggcgatg 180
agatccaagc ccactgtctg acaccagcag aaactgaagt gactgagtgt gctggcattc 240
tgaaagatgt tctgtcaaaa aaccttcatg agcttcaagg gctctgtaat gttaagaaca 300
aaatgggtgt accatgggtt tctgtggaag agctgggtca agagattata acaggcagat 360
tgccattccc ctctgtgggt ggcacaccag tgaatgacct ggtacgagtt ttggtggtgg 420
cagaatcaaa caccccagaa gagactccag aggaggagtt ttatgcctat gtggaacttc 480
agactgagtt gtacaccttt ggtctgtctg atgacaatgt ggttttcact agcgattata 540
tgactgtgtg gatgattgac attcccaaat cttatgtgga tgtgggcatg ctcacacgag 600
caactttcct ggagcagtgg ccgggtgcca aagtgactgt catgattccc tactcctcca 660
cctttacctg gtgtggagag cttggcgcca tctctgaaga atctgcccca caaccaagtt 720
tgagtgccag atccccggtc tgtaagaaca gtgccagata ttctacctca aaattttgcg 780
aggtagatgg atgcacagct gaaactggca tggaaaagat gtctctgctc actccatttg 840
gaggcccacc tcaacaggct aagatgaaca cttgcccatg ctactacaag tacagcgtga 900
gcccactgcc tgccatggac cacttgatcc tagctgacct agctggcctg gattctctca 960
cttcacctgt ttatgtgatg gcagcatatt ttgattctac acatgaaaat cctgtgagac 1020
catccagcaa gctttatcat tgtgctctac agatgacaag tcatgatggg gtgtggactt 1080
caacttccag tgagcagtgt cccattagac tggttgaggg ccagtctcaa aatgtgctgc 1140
aggtccgggt ggctccgaca tcgatgccta acctggttgg ggtcagcttg atgttggagg 1200
ggcaacagta caggctggag tattttgggg atcattgata ctcagtaact taagaggaaa 1260
ttttttgtt aaaaataaag ttatttt 1287

<210> 2
<211> 1218
<212> DNA
<213> Gamma herpesvirus

<400> 2
atggccttcc tatccacatc tgtgctcatt aaatgctgca tcctcctgtt ggcaggagga 60

```
ttggctgaga gccttactct aggtttggca cctgctctgt ctacccactc ttctggagtg 120
tcaacacagt ctgttgattt gtcacaaatt aaaagaggcg atgagatcca agcccactgt 180
ctgacaccag cagaaactga agtgactgag tgtgctggca ttctgaaaga tgttctgtca 240
aaaaaccttc atgagcttca agggctctgt aatgttaaga acaaaatggg tgtaccatgg 300
gtttctgtgg aagagctggg tcaagagatt ataacaggca gattgccatt ccccctctgtg 360
ggtggcacac cagtgaatga cctggtacga gttttggtgg tggcagaatc aaacacccca 420
gaagagactc cagaggagga gttttatgcc tatgtggaac ttcagactga gttgtacacc 480
tttggtctgt ctgatgacaa tgtggttttc actagcgatt atatgactgt gtggatgatt 540
gacattccca aatcttatgt ggatgtgggc atgctcacac gagcaacttt cctggagcag 600
tggccgggtg ccaaagtgac tgtcatgatt ccctactcct ccacctttac ctggtgtgga 660
gagcttggcg ccatctctga agaatctgcc ccacaaccaa gtttgagtgc cagatccccg 720
gtctgtaaga acagtgccag atattctacc tcaaaatttt gcgaggtaga tggatgcaca 780
gctgaaactg gcatggaaaa gatgtctctg ctcactccat ttggaggccc acctcaacag 840
gctaagatga acacttgccc atgctactac aagtacagcg tgagcccact gcctgccatg 900
gaccacttga tcctagctga cctagctggc ctggattctc tcacttcacc tgtttatgtg 960
atggcagcat attttgattc tacacatgaa aatcctgtga gaccatccag caagctttat 1020
cattgtgctc tacagatgac aagtcatgat ggggtgtgga cttcaacttc cagtgagcag 1080
tgtcccatta gactggttga gggccagtct caaaatgtgc tgcaggtccg ggtggctccg 1140
acatcgatgc ctaacctggt tggggtcagc ttgatgttgg aggggcaaca gtacaggctg 1200
gagtattttg gggatcat                                              1218
```

<210> 3
<211> 406
<212> PRT
<213> Gamma herpesvirus

<400> 3

```
Met Ala Phe Leu Ser Thr Ser Val Leu Ile Lys Cys Cys Ile Leu Leu
 1               5                  10                  15
Leu Ala Gly Gly Leu Ala Glu Ser Leu Thr Leu Gly Leu Ala Pro Ala
            20                  25                  30
Leu Ser Thr His Ser Ser Gly Val Ser Thr Gln Ser Val Asp Leu Ser
        35                  40                  45
Gln Ile Lys Arg Gly Asp Glu Ile Gln Ala His Cys Leu Thr Pro Ala
    50                  55                  60
Glu Thr Glu Val Thr Glu Cys Ala Gly Ile Leu Lys Asp Val Leu Ser
65                  70                  75                  80
Lys Asn Leu His Glu Leu Gln Gly Leu Cys Asn Val Lys Asn Lys Met
                85                  90                  95
Gly Val Pro Trp Val Ser Val Glu Glu Leu Gly Gln Glu Ile Ile Thr
            100                 105                 110
Gly Arg Leu Pro Phe Pro Ser Val Gly Gly Thr Pro Val Asn Asp Leu
        115                 120                 125
Val Arg Val Leu Val Val Ala Glu Ser Asn Thr Pro Glu Glu Thr Pro
    130                 135                 140
Glu Glu Glu Phe Tyr Ala Tyr Val Glu Leu Gln Thr Glu Leu Tyr Thr
145                 150                 155                 160
Phe Gly Leu Ser Asp Asp Asn Val Val Phe Thr Ser Asp Tyr Met Thr
                165                 170                 175
Val Trp Met Ile Asp Ile Pro Lys Ser Tyr Val Asp Val Gly Met Leu
            180                 185                 190
Thr Arg Ala Thr Phe Leu Glu Gln Trp Pro Gly Ala Lys Val Thr Val
        195                 200                 205
Met Ile Pro Tyr Ser Ser Thr Phe Thr Trp Cys Gly Glu Leu Gly Ala
```

```
    210                 215                 220
Ile Ser Glu Glu Ser Ala Pro Gln Pro Ser Leu Ser Ala Arg Ser Pro
225                 230                 235                 240
Val Cys Lys Asn Ser Ala Arg Tyr Ser Thr Ser Lys Phe Cys Glu Val
                245                 250                 255
Asp Gly Cys Thr Ala Glu Thr Gly Met Glu Lys Met Ser Leu Leu Thr
            260                 265                 270
Pro Phe Gly Gly Pro Pro Gln Gln Ala Lys Met Asn Thr Cys Pro Cys
        275                 280                 285
Tyr Tyr Lys Tyr Ser Val Ser Pro Leu Pro Ala Met Asp His Leu Ile
    290                 295                 300
Leu Ala Asp Leu Ala Gly Leu Asp Ser Leu Thr Ser Pro Val Tyr Val
305                 310                 315                 320
Met Ala Ala Tyr Phe Asp Ser Thr His Glu Asn Pro Val Arg Pro Ser
                325                 330                 335
Ser Lys Leu Tyr His Cys Ala Leu Gln Met Thr Ser His Asp Gly Val
            340                 345                 350
Trp Thr Ser Thr Ser Ser Glu Gln Cys Pro Ile Arg Leu Val Glu Gly
        355                 360                 365
Gln Ser Gln Asn Val Leu Gln Val Arg Val Ala Pro Thr Ser Met Pro
    370                 375                 380
Asn Leu Val Gly Val Ser Leu Met Leu Glu Gly Gln Gln Tyr Arg Leu
385                 390                 395                 400
Glu Tyr Phe Gly Asp His
                405
```

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/25871

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.
US CL : 424/86.1. 147.1; 435/5, 7.8; 530/387.9; 536/23.1, 24.32
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/86.1. 147.1; 435/5, 7.8; 530/387.9; 536/23.1, 24.32

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN/CAS: medline, caplus, embase, biosis.
WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	SIMAS. J.P. ET AL. Analysis of murine gammaherpesvirus-68 transcription during lytic and latent infection. Journal of General Virology. 1999. Vol. 80, pages 75-82.	40-48 --------- 49
X ---- Y	VIRGIN, H.W. ET AL. Complete Sequence and Genomic Analysis of Murine Gammaherpesvirus 68. Journal of Virology. August. 1997. Vol. 71, No. 8, pages 5894-5904.	40-48 -------- 49
X --- Y	VAN BERKEL, V. ET AL. Identification and Initial Characterization of the Murine Gammaherpesvirus 68 Gene M3, Encoding an Abundantly Secreted Protein. Journal of Virology. May, 1999. Vol. 73. No. 5, pages 4524-4529.	40-48 -------- 49

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
03 NOVEMBER 2000

Date of mailing of the international search report
27 DEC 2000

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
AMY DECLOUX
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/25871

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	VIRGIN, H.W. ET AL. Three Distinct Regions of the Murine Gammaherpesvirus 68 Genome Are Transcriptionally Active in Latently Infected Mice. Journal of Virology. March, 1999. Vol. 73. No. 3, pages 2321-2332.	40-48 -------- 49
A,P	VAN BERKEL, V. ET AL. Identification of a Gammaherpesvirus Selective Chemokine Binding Protein That Inhibits Chemokine Action. Journal of Virology. August, 2000. Vol. 74. No. 15, pages 6741-6747.	1-49

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/25871

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

A61K 39/12, 39/42; C07H 21/04; C07K 16/08; C12N 15/11; C12Q 1/70; G01N 33/53;

Form PCT/ISA/210 (extra sheet) (July 1998)★